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LONG-TERM EFFECTS OF PRE- AND POSTNATAL GLUCOCORTICOID TREATMENT IN CONGENITAL ADRENAL HYPERPLASIA

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Karolinska Institutet

Stockholm 2019

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Printed by Eprint AB 2018
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ISBN 978-91-7831-358-7

Long-term Effects of Pre- and Postnatal Glucocorticoid Treatment in Congenital Adrenal Hyperplasia

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Friday the 8th of March, 9:00 a.m.

Karolinska University Hospital, Skandiasalen (QA:01)

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Abstract

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder mostly caused by mutations in the *CYP21A2* gene leading to impaired production of cortisol and aldosterone. Precursors in the steroidogenic pathway are shunted to pathways of androgen production and elevated levels of androgens may cause virilization of the external genitalia in females with CAH already *in utero*. Prenatal treatment with the synthetic glucocorticoid (GC) dexamethasone (DEX) can ameliorate virilization of the female fetus but because of the recessive mode of the inheritance of CAH and that treatment has to be initiated before the genotype of the fetus can be determined, the majority of the treated cases will be unnecessarily exposed to DEX during fetal life. Moreover, patients with CAH require GC replacement therapy after birth and during their life span there may be episodes of over- or under-treatment with a risk of developing adverse effects. Side effects of pre-and postnatal GC exposure may develop into chronic conditions with permanent effects on growth, metabolism, cognition, behavior and normal immune functioning. In this study, the effects of prenatal DEX treatment and postnatal GC treatment in the context of CAH were evaluated in a cohort of 265 individuals. The cohort comprised DEX-treated individuals with and without CAH, patients with CAH not prenatally treated with DEX and controls from the general population. The long-term impact on cognition, behavior, brain morphology, metabolism and DNA methylation was studied.

Prenatal treatment with DEX was associated with cognitive impairments, particularly working memory. The effects seem to normalize by adult age in individuals without CAH who were treated with DEX during the first trimester of fetal life. In patients with CAH, prenatal DEX therapy was associated with reduced thickness and surface area bilaterally of a large area encompassing the parietal and superior occipital cortex. Moreover, the effects of DEX treatment on DNA methylation were associated with alterations in the DNA methylation profile, denoting an altered epigenetic programming of the immune system and, in particular, inflammation in individuals without CAH treated in the first trimester. This finding may confer altered risks for immune-related disorders later in life. When looking at the long-term outcome in patients with CAH, patients showed deficits in tests measuring executive functioning. Deficits in spatial working memory were associated with decreased white matter integrity that, in turn, was associated with lower dosages of GCs. Patients also showed structural alterations in the prefrontal regions involved in executive functioning and in areas of the parietal and superior occipital cortex involved in sensory integration. In addition, patients exhibited reduced cerebellar volume. In our analysis of DNA methylation in patients with CAH, we identified hypermethylation in two CpGs in two genes (*FAIM2* and *SFI1*). Methylation was associated with the severity of CAH and brain structure, but we could not identify any association between methylation in these two genes and metabolic or cognitive outcome.

In conclusion, this study extends our knowledge about the effects of pre-and postnatal GC treatment in CAH. The results have implications for the use of prenatal DEX treatment.

List of scientific papers

I. **Leif Karlsson**, Anton Gezelius, Anna Nordenström, Tatja Hirvikoski, Svetlana Lajic. Cognitive impairment in adolescents and adults with congenital adrenal hyperplasia. Clinical Endocrinology (Oxf) 2017(87):651-659

II. **Leif Karlsson**, Anna Nordenström, Tatja Hirvikoski, Svetlana Lajic. Prenatal dexamethasone treatment in the context of at risk CAH pregnancies: Long-term behavioral and cognitive outcome. Psychoneuroendocrinology 2018; 91:68-74

III. **Leif Karlsson**, Michela Barbaro, Ewoud Ewing, David Gomez-Cabrero, Svetlana Lajic; Epigenetic Alterations Associated With Early Prenatal Dexamethasone Treatment, Journal of the Endocrine Society, Volume 3, Issue 1, 1 January 2019, Pages 250–263

IV. **Leif Karlsson**, Michela Barbaro, Ewoud Ewing, David Gomez-Cabrero, Svetlana Lajic. Genome-wide investigation of DNA methylation in relation to cognitive and metabolic outcome in patients with CAH. (Manuscript)

V. Annelies van't Westeinde, **Leif Karlsson**, Malin Thomsen Sandberg, Anna Nordenström, Nelly Padilla, Svetlana Lajic. Differences in grey matter structure and white matter integrity in patients with congenital adrenal hyperplasia: Relevance for working memory capacity. (Manuscript)

Additional Publications (Not included in the thesis)

Débora de Paula Michelatto, **Leif Karlsson**, Ana Letícia Gori Lusa, Camila D'Almeida Mgnani Silva, Linus Joakim Östberg, Bengt Persson, Gil Guerra-Júnior, Sofia Helena Valente de Lemos-Marini, Michela Barbaro, Maricilda Palandi de Mello, Svetlana Lajic. Functional and Structural Consequences of Nine CYP21A2 Mutations Ranging from Very Mild to Severe Effects. International Journal of Endocrinology 2016; 2016:4209670

Lena Wallensteen, **Leif Karlsson**, Valeria Messina, Anton Gezelius, Malin Thomsen Sandberg, Anna Nordenstrom, Tatja Hirvikoski, Svetlana Lajic. Evaluation of behavioral problems after prenatal dexamethasone treatment in Swedish children and adolescents at risk of congenital adrenal hyperplasia. Hormones and Behavior 2018; 98, 219-224

Svetlana Lajic, **Leif Karlsson**, Anna Nordenstrom. Prenatal Treatment of Congenital Adrenal Hyperplasia: Long-Term Effects of Excess Glucocorticoid Exposure. Hormone Research in Paediatrics 2018; 89:362-371

CONTENTS

List of abbreviations

450K	Illumina Infinium Human Methylation450 BeadChip array
ACTH	Adrenocorticotropic hormone
AD	Axial diffusivity
ARMD	Age-related macular degeneration
ANOVA	Analysis of variance
AQ	Autism quota scale
CAH	Congenital adrenal hyperplasia
CBG	Corticosteroid-binding globulin
DTI	Diffusion tensor imaging
DMP	Differentially methylated probe
FA	Fractional Anisotropy
FDR	False discovery rate
FSIQ	Full scale IQ
GAT	Genetic association tester
GC	Glucocorticoid
GM	Grey matter
GO	Gene ontology
GR	Glucocorticoid receptor
GREAT	Genomic regions enrichment of annotations tool
GWAS	Genome-wide association studies
HC	Hydrocortisone
HPA	Hypothalamic-pituitary-adrenal
IBD	Inflammatory bowel disease
ISB	Iron status biomarkers
MD	Mean diffusivity
MDD	Major depressive disorder
MPV	Mean platelet volume
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PNMS	Prenatal maternal stress
RD	Radial diffusivity
SNP	Single nucleotide polymorphism
TBSS	Tract-based spatial statistics
TFCE	Threshold-free cluster enhancement
TSS	Transcriptional start site
WAIS	Wechsler adult intelligence scale
WISC	Wechsler intelligence scale for children
WM	White matter
WMS	Wechsler memory scale
OR	Odds ratio
ROI	Region of interest
UTR	Untranslated region

1 INTRODUCTION

1.1 CONGENITAL ANDRENAL HYPERPLASIA

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders characterized by impaired adrenal cortisol synthesis. The majority of cases with CAH have mutations in the steroid 21-hydroxylase gene (*CYP21A2*) (incidence 1:9800 in Sweden), which leads to an enzyme block in the biosynthesis of both cortisol and the salt-retaining hormone aldosterone. Subsequently, the accumulation of corticosteroid precursors will be shunted to the androgen-producing pathway leading to various degrees of hyperandrogenic symptoms. Depending on the genotype, the severity of the phenotype ranges from mild/late-onset/non-classic (NC) CAH to the more severe classic form with or without salt-wasting (SW) (simple virilizing, SV or SW CAH) A newborn child with SW CAH will die in circulatory shock because of an adrenal salt-losing crisis during the first weeks of life if glucocorticoid (GC) replacement therapy is not initiated Moreover, girls affected with classic CAH are born with virilized external genitalia due to the excess of androgens produced by the adrenal cortex, sometimes to such an extent that sex assignment of the new-born child may be difficult to ascertain [1-3].

In more than 95% of all patients with CAH, 10 common mutations are usually identified as the cause; however, over 200 *CYP21A2* mutations have been identified thus far. In CAH, there is generally a good correlation between genotype and phenotype, with the mildest mutated allele determining the severity of the disorder [4-6]. There is some variability, however: for example, patients with the I2splice mutation may develop either SV or SW CAH [6]. For more rare mutations, large groups of patients are not available for clinical investigation. In these cases, *in vitro* analysis can be used as a complement in disease classification [7-9].

Postnatal treatment for patients with CAH constitutes life-long GC replacement therapy with an attempt to mimic the physiological levels following the circadian rhythm. If the child has the classic form of CAH, substitution with fludrocortisone may be necessary to prevent a salt-losing crisis. Furthermore, neonates with SW CAH are at risk of circulatory shock during the first weeks of life if GC replacement therapy is not instituted [1]. For this reason, several countries, including Sweden, have introduced neonatal screening programs for CAH.

It is difficult to achieve perfect dosing of GC replacement therapy to precisely mimic the circadian rhythm of cortisol release, leading to a risk of over- or undertreatment during the person’s lifespan. In both situations, there may be adverse effects on the health of the patient over time [1, 10]. For example, overtreatment of patients with CAH may result in cushingoid features, obesity, suppressed growth with compromised final height and osteoporosis, insulin resistance and altered glucose tolerance. Undertreatment may lead to adrenal crises, accelerated bone age and hyperandrogenic symptoms [10]. The additive negative effect of

salt-losing crises and hypoglycemic episodes that may result from suboptimal treatment may also contribute to the long-term outcome of patients with CAH [11].

There are also differences between subgroups in CAH in patient outcome. In Swedish follow-up studies, the null genotype group (without residual enzyme activity), who is the most severely affected group, differ from the patient group with the I2 splice mutation when investigating long-term outcome, both in the outcome of genital surgery and psychological aspects [12-16]. Women with CAH may also suffer negative effects in sexual function and reproductive health, especially in the most severe cases [12, 13, 16]. This event may stem from the degree of virilization of the external genitalia, as well as a combination of other factors related to CAH [12, 13, 16]. Lastly, patients with the most severe genotypes have higher cardiovascular and metabolic morbidity [15].

1.2 PRENATAL DEXAMETHASONE THERAPY: AN ETHICAL DILEMMA

Prenatal virilization of girls with CAH can be minimized by silencing the fetal adrenal androgen production through exposure to dexamethasone (DEX), a synthetic GC. The treatment has been in use worldwide since the mid-1980s and has been shown to be effective in reducing or even preventing prenatal virilization. Treatment can be offered to expecting mothers who previously had a child with classic CAH and which is expected to result in severe virilization in girls (Figure 1) [17].

The treatment protocol is presented in Figure 1. The treatment has to be initiated before gestational week 7 to effectively prevent the closure of the labio-scrotal folds and the formation of the urogenital sinus. At this stage the genotype or the sex of the fetus is not known. When the results of the chorionic villous sampling are available (around GW 12-14) treatment is terminated in case the fetus is not affected by CAH or if the fetus is a male [17]. Girls with CAH are treated until term [18, 19]. Due to the recessive mode of inheritance and that only girls are virilized, 1 of 8 fetuses will benefit from DEX treatment and 7 of 8 fetuses will unnecessarily be exposed to excessively high doses of GCs during early embryonic life. It has been shown that early fetal sex typing using cell-free fetal DNA from maternal blood can be used to avoid prenatal treatment in boys, if done after 4.5 weeks of gestation. However, although unaffected boys can be excluded using this methodology, unaffected girls cannot be segregated from affected girls [20]. New *et al.* succeeded in treating only the affected female fetuses using massive parallel sequencing of cell-free fetal DNA derived from maternal blood [21]. This approach, however, requires expensive equipment and experienced personnel and is currently not part of routine clinical care.

This circumstance creates an ethical dilemma given that evidence suggests that disturbances in the hormonal and nutritional environment *in utero* may create a predisposition to disease later in adult life (the Barker hypothesis) [22]. Normally, the fetus is protected from excess GC exposure by inactivation of cortisol by the enzyme 11-beta-HSD type 2 (HSD11B2) in the placenta, resulting in fetal cortisol levels being about 1/5 to 1/10 of the maternal levels [23]. However, because HSD11B2 cannot inactivate DEX, which then can freely pass the

placenta, the dose used in the prenatal treatment of CAH will result in GC levels in the fetus that are estimated to be 30-60 times higher than normal [13, 24].

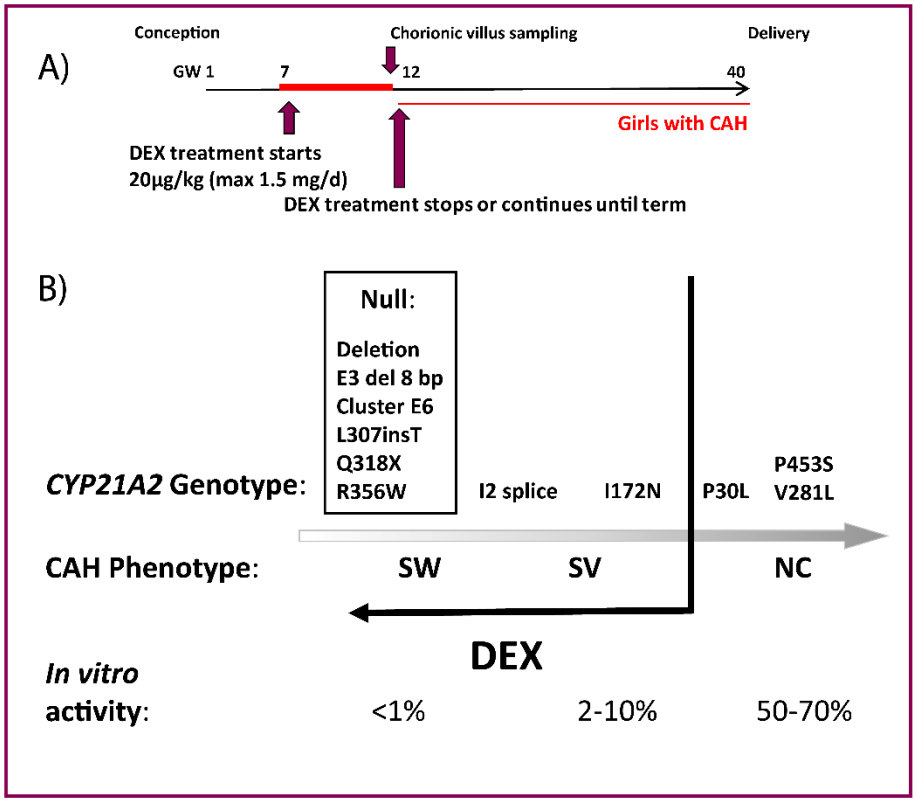

Figure 1. *A) Treatment protocol for prenatal dexamethasone therapy in pregnancies at risk of CAH. B) CYP21A2 variants that give rise to CAH in relation to clinical severity and enzyme activity assessed with in vitro studies. A group of null mutations, together with the intron 2 splice and I172N mutations, is associated with salt-wasting or simple virilizing CAH. Prenatal DEX treatment is restricted to families segregating these mutations. Figure adapted from Lajic et al. 2018 [25].*

GCs are important during fetal life for the differentiation and maturation of tissues. This feature of GCs is used in the treatment of pregnancies at risk of preterm delivery to induce, for example, pulmonary maturation and prevent intra-ventricular cerebral hemorrhage. Moreover, GCs affect fetal growth, resulting in lower birth weight and inhibition of neuronal proliferation [26, 27]. The effects of GCs on fetal development are time- and dose-dependent with different outcomes in early versus late gestational treatment [28, 29]. A study with full-term children treated prenatally with GCs showed an increased cortisol response to psychosocial stress compared with untreated children, indicating an altered programming of the hypothalamic-pituitary-adrenal (HPA) axis, with a greater effect seen in girls [30]. Cognitive functions have also been studied in preterm infants treated with synthetic GCs [31]. But because preterm birth may affect outcome as well, it is difficult to define the exact cause of any negative effects [31]. Moreover, prenatal DEX treatment may also affect the ovaries of

female fetuses: incubating human fetal ovaries 8-11 weeks post-conception with clinically relevant doses of DEX reduced the number of germ cells, which was caused by an increased rate of apoptosis [32].

There are also differences in the vulnerability to GCs that are due to genetic differences in the enzyme 11-beta-HSD type 1, the GC receptor and sex differences (girls being more sensitive than boys). Altogether, it makes the task to further assess the effects from prenatal DEX more complicated and difficult [30, 33, 34]. It is also important to be aware of the fact that it is difficult to distinguish between effects due to prenatal treatment versus postnatal treatment, which all patients with CAH receive.

The use and ethics of prenatal treatment in the context of CAH have been intensely debated during the past decades because of the inherent uncertainties with the treatment and that the majority of the treated cases do not benefit from the treatment at all. An international consensus was reached in the early 2000s that prenatal DEX treatment should only be offered within the frames of a clinical study and with explicit informed consent of the couple [35]. In Sweden, prenatal DEX treatment has been employed since 1985 and since 1999 as a clinical trial (PREDEX, PI, S Lajic) [36].

1.3 THE PHYSIOLOGY OF GLUCOCORTICOIDS

1.3.1 Synthesis, release and regulation of cortisol

GCs, mainly cortisol, are produced and secreted by the cells of the *zona fasciculata* in the adrenal cortex in response to adrenocorticotropic hormone (ACTH) from the pituitary. The production and secretion are regulated by the inhibitory effect of cortisol on both the hypothalamus and pituitary forming the HPA axis.

GCs exert their effects by binding to GC and mineralocorticoid (MR) receptors. Both receptors are expressed widely throughout the body and are mainly located in the cytosol of the cell, activating when the ligands diffuse into the cell and bind to the receptor. Upon binding, the receptors translocate to the nucleus, where they bind to DNA and subsequently regulate gene expression by either enhancing or suppressing gene transcription. The MR has a 10-fold higher affinity for GC than for GR and is presumably the main receptor used under basal conditions. The GR function would therefore be more important during increased cortisol levels, such as during the circadian peak given that the negative feedback of the HPA axis is primarily via the GR and the normal proactive effect is mediated through the MR [37-39].

In the periphery, levels of GCs are also regulated by the local metabolic conversion between the active and inactive forms. The enzymes 11β-hydroxysteroid dehydrogenase type 1 and 2 perform this action to prevent overstimulation of the MR by GCs in MR-containing targets (such as the epithelial cells in the kidney). Finally, another important factor of GC regulation is the corticosteroid-binding globulin (CBG) and serum albumin, which bind and deactivate

circulating free GCs. Only 5% of the GC is normally metabolically active and thus not bound to CBG/albumin [40-42].

1.3.2 Effects of glucocorticoids in the human body

1.3.2.1 Metabolic and Cardiovascular Effects

GCs have an important glucose-sparing effect for human glucose metabolism as they stimulate glycogen formation, especially in the liver [43]. In response, adipose tissue releases fatty acids into the blood while other tissues switch to break down fatty acids and proteins instead of glucose [43]. This mechanism is important during fasting or other catabolic states. Excessive exposure to GCs leads to a decrease in proteins in muscle, bone, connective tissue and skin and an increase in blood sugar and blood lipids. GCs also counteract insulin action causing hyperglycemia, which may develop insulin resistance with time [43-45]. Moreover, insulin resistance may also be the result of hyperlipidemia and lipodystrophy, among other factors [43, 45] (both may be caused by GC exposure). Therefore, chronic exposure to GCs carries the risk of causing insulin resistance by impairing normal metabolism and insulin action [43]. However, it has also been hypothesized that inflammation may cause insulin resistance [45]. Consequently, altered levels of GCs may contribute to the development of insulin resistance through their anti-inflammatory characteristics [45].

Moreover, GCs participate in regulation of blood pressure by contributing to the responsiveness of vascular smooth muscle to catecholamines that constrict the arterioles and decrease the effects of prostaglandins that induce vasodilation. In addition, GCs affect myocardial contraction and underproduction of cortisol leads to hypotension. In the kidneys, GCs increase the glomerular filtration and are essential for the rapid excretion of water load. Cortisol also has a negative effect on the kidneys' response to antidiuretic hormone [40, 41, 46].

1.3.2.2 Effects on the Immune System

Because they inhibit the production of pro-inflammatory cytokines and generation of eicosanoids (such as prostaglandins and leukotrienes), which promote vascular dilatation and permeability during inflammation, GCs exhibit anti-inflammatory effects. GCs also decrease blood flow to inflammatory sites by sensitizing endothelial cells to vasoconstrictors, and they attenuate leukocyte recruitment to inflammatory sites by inhibiting production of chemokines, chemo-attractants and leukocyte-expressed adhesion molecules [47]. Finally, mast cells exposed to GCs are less likely to release histamine and other pro-inflammatory substances [40].

Additional to their anti-inflammatory effects, GCs are important for T-helper cell activation by inhibiting the antigen-presenting capabilities of dendritic cells. Finally, GCs affect T-cell activation by interfering with T-cell receptor signaling [47].

1.3.2.3 Effects on the Central Nervous System

Apart from their metabolic effects, GCs may have an effect on the CNS through disruption of neuronal energy metabolism. GCs are involved in the regulation of the HPA axis through the actions of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). The GR is ubiquitously expressed throughout the brain, whereas the MR is mainly expressed in the limbic structures. Neurons within the amygdala, hippocampus and prefrontal cortex co-express both MR and GR at high levels [48-50]. The amygdala, hippocampus and prefrontal cortex, important for emotional regulation, memory functioning and executive functioning, respectively [51-53], are vulnerable to high doses of GCs. In humans, memory deficits have been found in conditions characterized by prolonged exposure to elevated GCs, such as Cushing's syndrome [54] and in individuals receiving GC treatment [55, 56]. The exact pathophysiological mechanisms of how GCs affect cognitive function and CNS structure is not known. However, there are several proposed mechanisms, of which most have only been studied in animals [57]. Some key features will be discussed here.

The direct effects of GCs on neurogenesis have been extensively investigated in rodents. Although an exact mechanism cannot be identified, a conclusion that can be extrapolated from these studies is that GCs inhibit neurogenesis through activation of the GR [57]. In contrast, activation of the MR enhances neurogenesis and cell proliferation [57]. This observation is in line with the given affinity differences between MR and GR for GCs: excessively high GC levels via GR activation are detrimental to neurogenesis, whereas physiological GC levels have a positive effect on neurogenesis via MR activation.

GCs also affect CNS structures and cognition by affecting basic neurotransmitter systems. For example, GCs are known to increase expression of the serotonin receptor and transmitter uptake [58, 59], and during periods of stress, the dopamine levels increase in key structures and interacting areas of the limbic system, the hippocampus, amygdala and prefrontal cortex [60]. Alterations of these transmitter systems may subsequently be attributed to, for example, cognitive deficits and/or structural alterations [57, 61]. Other neurotransmitters, such as glutamate, GABA, acetylcholine and noradrenaline, play an important role in mediating stress responses [57, 60] and may be of relevance for outcome following GC treatments. GCs may also affect neuronal firing by directly increasing calcium currents and thereby indirectly increasing calcium-dependent potassium currents in neurons [62-65].

There is also evidence that inflammation decreases the rate of neurogenesis, and consequently, pro-inflammatory cytokines have been implicated in the mediation of stress effects. As previously mentioned, GCs have a significant role in mediating anti-inflammatory processes [57]. Thus, as a secondary effect, GCs could, via an altered immune response in response to stress, promote neurogenesis by inhibiting the production of pro-inflammatory cytokines. This possibility highlights the important relationship between the CNS and the immune system relative to the effect of GCs on both systems. Neurogenesis may further be affected by differential effects of stress on neurotrophic factors, such as brain-derived neurotrophic factor *(BDNF)* and vascular endothelial growth factor (*VEGF)* [57]. Levels of

BDNF and *VEGF* decrease under stress and this, in turn, reduces cell differentiation and proliferation as a result [57].

1.4 EFFECTS OF EXCESSIVE GLUCOCORTICOID EXPOSURE

1.4.1 Cognitive and behavioral effects of GC exposure

Large amounts of data are available on the effects of GCs on human cognition. The effects are dependent on the timing, length, magnitude and mode of exposure. In Canada, a study with children exposed to prenatal maternal stress (PNMS) during a natural disaster, the 1998 ice storm, reported that exposure to any level of PNMS during early pregnancy is associated with poorer temperament in infants [66]. Furthermore, at 5½ years of age, children exposed *in utero* to high levels of objective stress had lower Full Scale IQ (FSIQ), poorer verbal intelligence and lower language abilities compared with children exposed to lower levels of PNMS [67]. Moreover, prenatal treatment with synthetic GC during the third trimester has been shown to be associated with negative effects on mental health in childhood and adolescence. This observation was detected as general psychiatric disturbance, inattention and antisocial behavior at 8 years of age in a Finnish study in which the children were assessed by their teachers [68].

In a study from the Netherlands comparing GC treatments used to prevent bronchopulmonary dysplasia, effects on preterm children, including untreated, hydrocortisone (HC)-treated and DEX-treated children, DEX was shown to have negative effects in girls [69]. Neonatal DEX treatment resulted in more social problems and more anxious/depressed behaviors in preterm girls. Of note, the scores in the neonatal DEX-treated girls were similar to those observed in untreated girls born preterm [69]. However, this result was not true for girls born preterm who were treated with HC postnatally [69].

In our Swedish cohort of prenatally DEX-treated children, we did not observe differences in parent-reported psychopathology or behavioral problems. However, DEX-treated children were reported to be more sociable by the parents, even though they scored higher in self-reported social anxiety [36, 70]. However, in our latest follow-up, the effect was no longer significant and the children seemed to be generally well adjusted [71]. Healthy children at risk of CAH who were treated during the first trimester with DEX exhibited deficits in cognitive functions (defined as lower performance in verbal working memory tasks) [70]. In our subsequent follow-up study, these effects seem to be sex-dimorphic, i.e. treated girls, but not boys, were affected. In addition to negative effects on executive functions, the girls had broader effects on cognition, as measured by lower test scores on tests assessing verbal and nonverbal intelligence [33].

1.4.2 Cognition, behavior and psychopathology in congenital adrenal hyperplasia

Because postnatal treatment for CAH consists of lifelong GC replacement therapy, it is important to discuss cognition and behavior in the context of CAH. Studies on intelligence in CAH have been inconsistent and contradictory. Some studies suggest that patients with CAH have lower FSIQ [11, 72], whereas in other studies general intelligence, irrespective of age, is not affected [73]. Still, patients with CAH have been found to have deficits in verbal working memory, as measured by the *Digit span* subscale from the Wechsler Adult Intelligence Scale-IV (WAIS-IV) [74], which, in turn, predicts poorer performance on spatial and arithmetic tasks [75, 76].

Because of the possibility that cognitive outcome may also be affected by the different clinical manifestations of CAH depending on the genotype of the patient, deficits in cognitive functions are not necessarily due to postnatal GC treatment of CAH [16, 77]. Women affected with SW CAH were less likely to complete their primary education in a Swedish epidemiological study. Moreover, both men and women had higher rates of disability pensions and sick leaves [16]. Investigations of the psychiatric morbidity in the same cohort indicate that women with CAH are at an increased risk of being diagnosed with a psychiatric disorder, including substance abuse, mood and anxiety disorders and stress and adjustment disorders [77]. A similar spectrum of psychiatric diagnoses was seen in men with CAH [78]. Other Swedish follow-up studies have shown that patients with CAH exhibit sex-atypical behavior, which affected quality of life in general. Women in the null genotype group were considerably more affected by the disease than women with other genotypes, including the I2 splice genotype group [12, 79]. Salt-losing crisis and hypoglycemia are also important factors that may contribute to the adversities in cognitive outcome seen in patients with CAH [11].

Another pertinent question when addressing cognitive functions and behavior in general in CAH is the potential programming effect of prenatal androgen exposure in affected girls [5, 75, 79]. In particular, there are profound effects on behavior in girls with CAH [5, 79], where they exhibit more male-like behaviors and altered preferences, indicating a masculinizing effect of the brain, probably stemming from prenatal androgen exposure [5, 79]. There is also evidence that women with CAH have greater spatial abilities in general as a consequence of the masculinization [80], although this finding is contradicted in some studies [75]. Speculatively, one might suggest that failure to detect effects in some cohorts may stem from the genetic background of the studied patients. This speculation is based on evidence that some effects are related to the severity of the mutation in combination with differences in the clinical management of CAH between countries [5, 79].

1.4.3 Structural effects on the CNS

Numerous studies described the negative effects of GC exposure on brain structure and function. Different experimental models for this purpose include the exposure to chronic

stress [81, 82], studies of patients with Cushing's syndrome [83] and pre-and postnatal GC treatment [84].

A follow-up study examining brain structures using magnetic resonance imaging (MRI) in GC-exposed children whose mothers were at risk of preterm delivery and therefore treated with betamethasone showed an 8% thinner rostral anterior cingulate cortex in the treated children [81]. Moreover, DEX given postnatally to extremely preterm babies resulted in smaller brain volumes at 18 years of age compared with same-aged children not subjected to DEX therapy [84].

A meta-analysis of MRI reports on Cushing's syndrome concluded that patients during periods of active disease have smaller hippocampal volumes, enlarged ventricles and cerebral atrophy [83]. One of the included studies in the meta-analysis could also identify smaller amygdala volumes in children during active disease [85]. The observed brain abnormalities could recover at least partially after correction of the hypercortisolism caused by the syndrome [83]. In contrast to these observations in Cushing's syndrome, chronically stressed-out women have larger amygdala volumes along with a reduced caudate volume and thinning of the medial prefrontal cortex, but no effect on hippocampal volume [82]. The increase in amygdala volume has also been observed in combat veterans with posttraumatic stress disorder (PTSD) [86]. The discrepant findings indicate that, in the context of GC effects on brain structure, the type and timing of exposure is of importance and modulates the outcome.

Concerning brain structure and CAH, very little is known about the long-term effects. There is only one study using a case-versus-control design with standardized software pipelines for analysis [87]. The authors of this study identified widespread reductions in white matter (WM) structural integrity and reductions of volumes in several brain regions, as well as a significant association between current GC replacement regimens and cognitive and CNS abnormalities. Regrettably, other available studies are mostly based on inspections of MRIs of the brain of single cases. However, as regards the available data, there are reports describing an increased incidence of WM abnormalities in patients with CAH [88-91].

1.4.4 DNA methylation and epigenetics

Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence but affect gene expression [92]. The most generally described epigenetic modifications of the genome are the modification of the N-terminal tails of histones and DNA methylation [92-95]. These modifications are important not only for the commitment of cells down specific differential paths but are also important for genomic integrity [92].

DNA methylation is the covalent attachment of a methyl group at DNA bases [93-95], mostly CpG sites in humans [93-95], and is an important and first identified epigenetic regulator [93-95]. It is involved in gene transcription, silencing (e.g., X-chromosome inactivation) and genomic imprinting [94, 95]. The process of establishing and maintaining DNA methylation

is performed by unique functions of a set of enzymes named DNA methyl transferases (DNMTs) [94, 95]: DNMT1, DNMT3A, DNMT3B and DNMT3L. The genomic profile of DNA methylation is set during embryogenesis and DNMTs are essential for this process, and consequently, very important for normal fetal development [94-97]. Before fertilization, however, oocytes and sperm differ in their respective methylation profile [98]. Sperm genomes are hypermethylated and tightly packed, whereas oocytes have a more open chromatin conformation [98]. After fertilization, the sperm methylomes are quickly erased and the oocytes supply new histones for the sperm genome [98]. Maternal DNA is also de-methylated but this seems to be a more passive process compared with sperm DNA [98]. During this process, information about imprinted genes are transferred, but how this works is not fully understood [98]. Genomic imprinting refers to the phenomenon that some genes are expressed in a monoallelic manner depending on the sex of the parental origin and is regulated by DNA methylation [98].

It has been shown that DNA methylation has different effects depending on the location of the methylated CpG site in relation to the gene. Hence, the location is crucial when studying DNA methylation [95]. For instance, gene promotor methylation is generally associated with gene silencing, whereas gene body methylation has been associated with activation through regulation of gene splicing [95]. DNA methylation has also been shown to have regulatory roles in intergenic features such as enhancers and insulators [95]. Importantly, to investigate DNA methylation regulation of gene expression, it is necessary to study the mechanisms regulating DNA methylation.

Epigenetic modification of DNA by methylation is a potential candidate for a mediating mechanism by which GCs could result in poor outcomes in the offspring through dysregulation of genes. This is also the working hypothesis of two of the projects included in the present thesis. The DNA methylation profile set during development does not remain stable throughout life. Moreover, the profile is highly tissue-specific [94-97] and changes in methylation have been associated with human disease [95]. For instance, alterations in DNA methylation have been observed in patients with type 2 diabetes [99]. These alterations may explain some of the underlying mechanisms in the pathogenesis of the disease and thereby explain part of the missing heritability [99]. Although other risk factors may affect DNA methylation (e.g., obesity), the observed changes still suggest an important role of epigenetic alterations for the disorder [99]. Moreover, peripheral DNA methylation has been associated with depression [100] and childhood abuse [101] in genes involved with stress, neural plasticity and brain circuitry. Furthermore, peripheral DNA methylation of the serotonin transporter gene is associated with functional activation during emotion processing [102]. In addition to suggesting an epigenetic mechanism as to brain function after exposure to stressors, these studies indicate that alterations of genes of interest in biological models may be detectable in the periphery [100-102].

In addition to disease, DNA methylation is susceptible to environmental changes, changes in physiological activity [103] and even to changes in the social environment [104, 105]. DNA

methylation also changes throughout an individual's lifespan as a function of aging [106, 107].

Finally, it is also important to consider that exposure to various factors during pregnancy may affect genomic imprinting [108, 109]. DNA methylation in infants whose mothers used folic acid supplements deviated in imprinted genes (*H19* and *IGF2*) and also caused deviation from the monoallelic expression [108]. Moreover, in a study in children and adolescents subjected to PNMS during the 1998 Quebec ice storm, broad changes in DNA methylation in peripheral T-cells were associated with the degree of stress exposure to the mother [109]. The changes were functionally organized and indicated an altered programming of the immune system [109]. Altered DNA methylation was further found to correlate with the levels of peripheral cytokines in the blood of the offspring [110]. The altered cytokine levels were subsequently attributed to a shift in the levels of Th1 cells towards Th2 cells [110, 111]. Together, these observations indicate that prenatal exposure may alter epigenetic programming that may be detected years after the initial exposure and possibly affect the individual's health outcome.

2 HYPOTHESIS AND AIMS

The overall aim of this thesis was to evaluate the effects of pre- and postnatal GC treatment in the context of CAH.

We hypothesized that:

1. Both pre- and postnatal GC treatment may have long-lasting effects on cognition and behavior (Papers I-II).

2. Early prenatal DEX treatment may have long-lasting effects in the epigenome of treated cases and that these alterations affect cognitive functions (Paper III).

3. Patients with CAH have a specific epigenomic profile that is linked to metabolic and cognitive outcome and that this profile may be different in CAH patients treated prenatally with DEX (Paper IV).

4. Pre- and postnatal GC treatment in patients with CAH alters brain structures in regions critical for executive functioning (Paper V).

3 METHODS AND MATERIALS

3.1 STUDY POPULATION

This thesis is part of a larger clinical study (PREDEX) evaluating the prenatal treatment of CAH in individuals at risk of CAH and treated prenatally with DEX. In total, PREDEX includes 265 individuals, see figure 2. Since 1984, 77 pregnancies have been treated with DEX in Sweden to avoid virilization in girls with CAH. The dose used is 20 µg/kg of the maternal pre-pregnancy weight and divided into 3 doses per day (maximum 1.5 mg/day). Treatment was offered to mothers who previously had a child with classic CAH and where the new pregnancy was expected to result in severe virilization in case the fetus was a girl. Four of the pregnancies resulted in miscarriages or termination. Thus, between 1984 and 2010, 73 cases in Sweden have received prenatal DEX treatment [33]. Four mothers were treated twice. Sixty of the children did not have CAH and 16 did, of whom 46 and 14, respectively, participated in the study. Thus, 60 DEX-treated participants were included in the study.

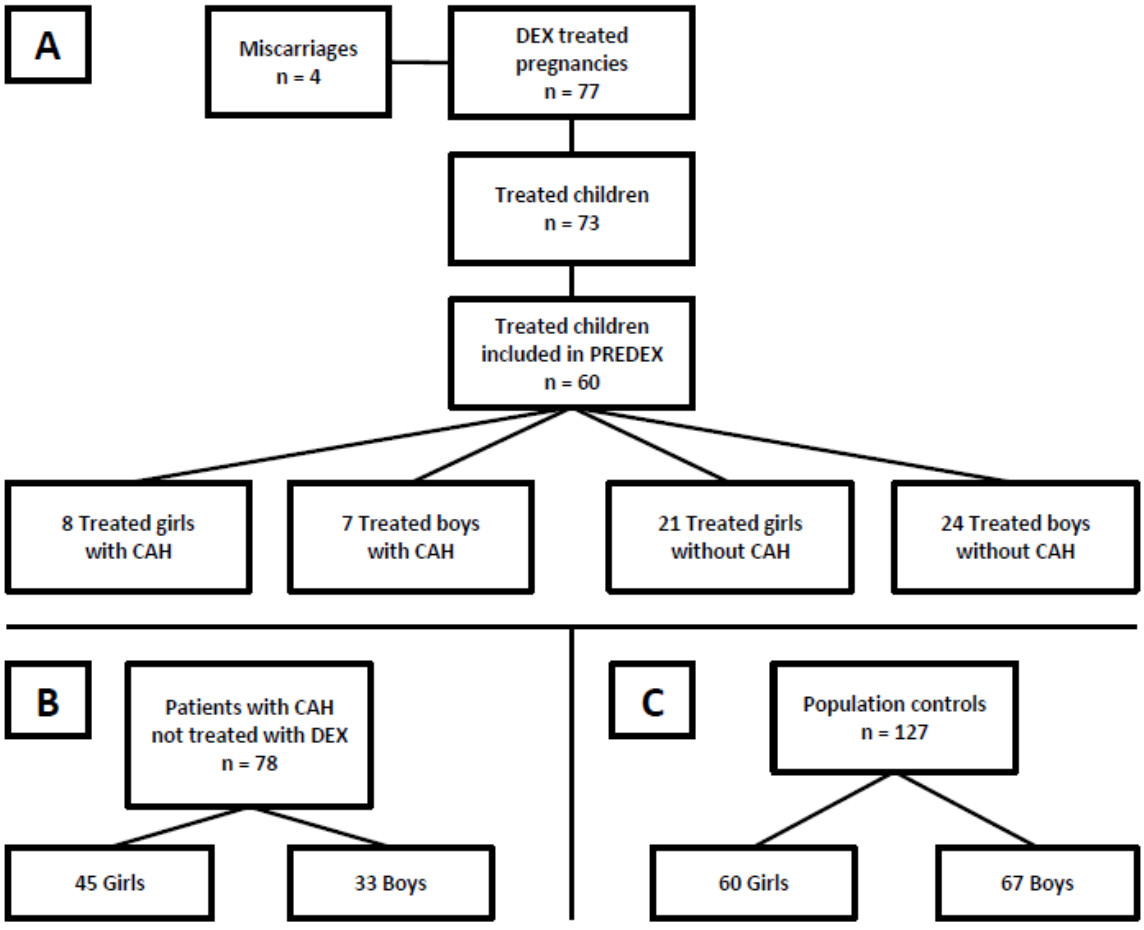

Figure 2. *Flowchart of the PREDEX cohort depicting; A) included DEX treated participants; B) patients with CAH not treated prenatally with DEX; C) population controls*

Furthermore, patients with CAH (n=77) who were not prenatally treated were included to investigate the long-term effects of CAH as well as the health effects of cortisol replacement

therapy but they also served as a control group to prenatally DEX-treated CAH patients. Population controls (n=127) were identified through the Swedish Population Registry and were matched on sex and age to the DEX-treated individuals and patients with CAH. They were randomly selected among individuals of the same sex and age in Stockholm County. All families/participants were initially contacted via an invitational letter and gave their written informed consent to participate in the follow-up studies. The studies were approved by the Regional Ethics Committee of Karolinska Institutet (dnr 99-153). The studies in the thesis were performed on subgroups derived from the main cohort and divided up into subgroups based on age. In papers I, II and V, participants ≥16 years were included while papers III-IV included both children and adults. All follow-up studies were performed at the Karolinska University Hospital.

3.1.1 Procedure

All participants were instructed not to eat after midnight the night before the visit to the Karolinska University Hospital. Blood samples for the methylation and analysis of glucose homeostasis and lipid profiles were collected in the morning. The participants' height and weight were measured immediately after sample collection. Blood (B) glucose, serum (S) Insulin, S-C-peptide, B-HbA1c, plasma (P) triglycerides, P-cholesterol, P-high-density lipoproteins (HDL) cholesterol and P-low-density lipoprotein (LDL) cholesterol were analyzed at the accredited clinical chemistry laboratory at the Karolinska University Hospital. The participants then completed a series of neuropsychological tests during one session and a series of brain imaging scans using MRI during a second session. Socioeconomic background, estimated as level of parental education, and data on participant education were collected. In addition, the participants were asked about their general wellbeing (using a continuous 10-point visual analogue scale, with 1 indicating the lowest score in wellbeing and 10 the highest), smoking behavior and drug and alcohol consumption.

3.2 ASSESSMENT OF COGNITION AND PSYCHOPATHOLOGY

In the studies comprising papers I and II, psychopathology, autistic traits and self-perceived executive dysfunctions were assessed with self-rating questionnaires. In the same studies, neuropsychological tests measuring general intelligence, executive functions and learning and memory functions in participants' ≥16 years were performed. Trained psychologists assessed all participants and the total time for the neuropsychological assessment was approximately one hour.

In total, 136 individuals were assessed: 23 DEX-treated participants without CAH, 9 DEX-treated patients with CAH, 46 prenatally untreated patients with CAH and 58 population controls. The positive response rate for the DEX-treated participants was 85.7%, 61.1% for patients with CAH and 26.3% for the population controls. The reasons for refusal are not known, but the length and complexity of the testing procedures could be one plausible explanation. Of the included patients with CAH, 76.4% were diagnosed through the national neonatal screening program for CAH and included 32 patients with SW CAH, 18 with SV

CAH and 5 with NC CAH. When dividing them further into genotype groups, 12 (22%) had the null genotype and 42 had a non-null genotype. The genotype for one woman with CAH was not known at the time of analysis. The type of GC substitution was known for 43 patients with CAH and, of these, 27 were treated with HC, 10 with prednisolone and 6 with a combination of HC and prednisolone; one patient received cortisone acetate for replacement.

The DEX-treated groups did not differ in socioeconomic background compared with controls as estimated by parental and participants level of education (all ps >0.05). However, the older group of patients with CAH (≥16 years, papers I and V) was, on average 3.7 years older than the population controls and had a higher level of education (both ps<0.05). All participants were between 16 and 33 years old.

3.2.1 Neuropsychological tests

General intelligence was estimated using two subtests from the Wechsler Adult Intelligence Scale-IV (WAIS-IV) [74]: *Matrices* to estimate nonverbal logical reasoning and *Vocabulary* to estimate verbal intelligence. Executive functions were estimated using the Wechsler Adult Intelligence Scales-IV (WAIS-IV) subtests: *Digit-span* (verbal working memory) and *Coding* (processing speed). Visual-spatial working memory was assessed using the *Span Board Forward/Backward* Test from the Wechsler Memory Scales-III (WMS-III) [112]. The Stroop color-word test was used to assess the ability to inhibit an overlearned response [113]. The *List learning* subtest from WMS-III [112] was used to measure learning and long-term memory. Lastly, all participants filled in the Barkley Deficit in Executive Functioning Scale – Short Form (B-DEFS-SF) [114].

3.2.2 Psychopathology and autistic traits

The Montgomery Åsberg Depression Ratings Scale (MADRS) [115] and the Hospital Anxiety and Depression Scale (HADS) [116] were used to assess depression. Liebowitz Social Anxiety Scale: Self Report (LSAS-SR) was used to assess social anxiety [117, 118]. The 10-item version of the self-report questionnaire, Autism Quota (AQ10) [119], was used to estimate autistic behaviors and traits.

3.2.3 Statistical analyses

Raw scores from neuropsychological tests were transformed before analysis into scaled scores (M=10, SD=3) based on age-specific Swedish norms for the included subtests from the Wechsler Scales (WAIS-IV and WMS-III) [74, 112]. There are currently no Swedish norms for the Stroop test and therefore raw scores were transformed into T scores (M=50, SD=10) according to American norms [113].

For comparison between groups (DEX versus controls, CAH versus controls) general two-way ANOVAs were performed that included the factors Group (CAH or DEX versus controls) and Sex (female, male) to compare the performances of the patients with CAH who were not prenatally treated with DEX or participants without CAH but treated prenatally with

DEX with those of the respective controls. All interactions ($p<0.1$) between group and sex were followed up by separate post hoc comparisons between patients and controls of the same sex to identify sex-specific effects. One-way ANOVAs were separately performed for different phenotypes (SW or SV) and genotypes (null or non-null) to investigate whether the severity of CAH was associated with cognitive performance. Moreover, the study investigated whether the GC replacement dose at the time of testing correlated with the estimates of cognitive functions in the non-DEX-treated CAH cohort. To achieve this, the medication dose was converted to HC equivalent in mg/m2 of body surface and correlated with measures of cognition using Pearson's bivariate correlation analysis.

Furthermore, in a subgroup of individuals without CAH but treated prenatally with DEX during the first trimester, we assessed their cognitive functions both during childhood and at adult age to investigate changes in cognitive functions over time (n=17) [33, 70]. To this end, cognitive performance in this subgroup assessed during childhood was compared with cognitive data assessed at adult age. The age-specific scaled scores from the Wechsler Intelligence Scales [120], subscales (*Matrices, Vocabulary, Digit span* and *Coding)* from the child (WISC) and adult (WAIS) versions and the Stroop test were used for this comparison. One-way within group ANOVAs with repeated measures were used to compare scores acquired in childhood with those acquired at adult age.

All analyses were conducted using SPSS 23 (IBM, Armonk, NY, USA) and a two-tailed alpha level of $p<0.05$ was adopted for all comparisons. Correction for multiple comparisons was not performed in order not to miss small, but potentially clinically relevant, effects. Effect sizes were calculated as Cohen's *d* [121].

3.3 DNA METHYLATION ANALYSIS

To study epigenetic programming effects, genome-wide DNA methylation was investigated in participants, allowing us to derive the effects of prenatal DEX and the effects of CAH. DNA methylation measurements were done using the Illumina Infinium HumanMethylation450 BeadChip array (450K). The 450K array was chosen as it has a genome-wide coverage and therefore is able to provide genome-wide methylation profiles for analyzed samples.

In total, 29 DEX-treated participants without CAH, 28 patients with CAH, 11 patients with CAH prenatally treated with DEX and 37 controls were included. The entire cohort of patients with CAH, including prenatally DEX-treated patients, consisted of 2 patients with NC CAH, 13 with SV CAH and 24 with the SW phenotype. There were no significant differences between groups for age. In addition, there were no differences in the daily GC dosages between prenatally untreated and prenatally treated patients with CAH. Participants were aged 5 to 29.6 years.

3.3.1 Isolation of T-cells

We chose to investigate DNA methylation in peripheral CD4+ T-cells because the tissue is easily accessible and because we could minimize the effect from having multiple cell types with different methylomes. Moreover, it is conceivable that GCs have very specific effects on T-cells based on their effect on the immune system. We may also use the cell type as a model to study mechanisms or events that may occur in other cell types during embryogenesis and postnatal development after GC exposure [122].

Each participant provided 50 ml blood in EDTA tubes, immediately followed by processing. The blood was transferred to 75 cm2 cell culture flasks (Falcon), diluted up to 100 ml in phosphate buffered saline (PBS) and distributed into sterile 50 ml tubes with porous barriers (LeucoSep). Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation on Ficoll-Plaque Plus at 800 g for 15 minutes (min). PBMCs were then washed three times with PBS before being counted and evaluated for viability using trypan dye exclusion. PBMCs were prepared for magnetic-activated cell sorting according to the manufacturer's instructions (Miltenyi Biotech). T-cells were purified from the PBMCs by positive selection using anti-CD4+ antibodies coupled to paramagnetic beads (Miltenyi Biotech). Cell separations were done on LS (Miltenyi Biotech) columns as per the manufacturer's instructions (Miltenyi Biotech). After separation, T-cells were counted and aliquoted to approximately 5 x10^6 per vial, snap frozen and stored at -80°C. A replicate of approximately 0.1 x 10^6 cells was taken for validation of cell population purity by flow cytometry. For a more detailed description of T-cell isolation and flow cytometry, see Reinius *et al*. [123]

3.3.2 Flow cytometry

The purity of CD4+ cell populations was verified using two-color antibody panels. Cells were re-suspended in PBS (0.1% bovine serum albumin). Fc receptors were blocked with a 10 µl FcR blocking reagent (Miltenyi Biotech) during 10 min at 4°C. Fluorochrome-conjugated anti-CD3 and anti-CD4 monoclonal antibodies were added to the cells for 10 min at 4°C. Every staining included unstained samples and isotype controls to set the gates for positive and negative populations. After staining, cells were washed and fixated in 1% formaldehyde in PBS. Data were acquired and analyzed using the Cyan ADP Analyzer (Summit 4.3, Beckman Coulter), with at least 5000 events per population.

3.3.3 DNA extraction, bisulphite treatment and DNA methylation measurements using the 450K BeadChip array

DNA was isolated from T-cell pellets using the QiAmp DNA Mini Kit (Qiagen) as specified in the manufacturer's instructions. DNA concentration was measured using the Qubit 2.0 (Invitrogen). Bisulphite treatment was performed with the EZ-96 DNA Methylation Kit (Zymo Research) and DNA methylation measurements were executed using the Illumina Infinium HumanMethylation450 BeadChip array (Illumina). The array was analyzed at BEA

- the core facility for Bioinformatics and Expression Analysis at Karolinska Institutet. Samples were analyzed in two batches and samples from patients with CAH, prenatally treated participants and controls were distributed randomly on the chips. This procedure was done to avoid effects of positioning bias of the samples.

3.3.4 Quality control and data processing

To estimate methylation levels, the 450k array measures the intensities of the methylated and unmethylated probes at the interrogated CpG site [124]. The 450k array was used to measure locus-specific DNA methylation levels at over 480 000 CpGs across the genome. All quality control, data processing and statistical analyses were performed in R. Raw data were pre-processed using the lumi package [124, 125]. After quality control had been applied, three controls and two DEX-treated participants without CAH were excluded because of a poor genome-wide correlation with other samples and an aberrant distribution of β values. β-values are a value between 0 and 1 and are calculated as the ratio of the methylated probe intensity and the overall intensity (sum of methylated and unmethylated probe intensities) [124].

Moreover, the following probes were excluded during the pre-processing of the analysis: (i) probes located on the Y and X chromosomes to remove the effect from having silenced X chromosomes in girls, (ii) probes with a single nucleotide polymorphism (SNP) located within three base pairs of the interrogated CpG site to exclude false positive probes caused by genetic variations and (iii) CpG probes with poor detection p-values ($p>0.01$) [126]. After filtering the data based on these criteria, 395 462 probes remained. β-values for the probes were estimated using a previously described three-step pipeline [124, 127]. Batch effects were identified and their effect quantified using principal component analysis and subsequently corrected using the ComBat function from the sva Bioconductor package [128].

3.3.5 Differential methylation analysis

A linear model was generated for each CpG site to identify differentially methylated probes (DMPs) for which the predictive variables for DNA methylation were group (CAH or DEX versus control) age, sex and group interaction with sex. Four analyses were conducted to evaluate the association between DNA methylation and DEX or CAH:

- One comparing first trimester DEX-treated participants to population controls
- One between patients with CAH (not prenatally treated) and population controls
- Two between prenatally DEX-treated patients with CAH and untreated patients with CAH (a separate analysis for each sex because of the difference in treatment length between sexes).

Based on the assumption that most of the CAH-associated DNA methylation changes would be relatively small and that, while using all available samples, our sample size of patients and controls was limited, only highly variable probes were analyzed. Probes were selected whose interquartile range, after transforming β-values into M-values, [124, 129], was >0.5. M-

values are calculated as the log2 ratio of the intensities of methylated probe versus unmethylated probe at the interrogated CpG site [124]. This procedure resulted in 29 351 probes selected for the association analysis. To estimate the significance of each probe for each respective analysis, a permutation-based p-value was computed in which 10 000 permutations were performed over the M-values for all probes. The false discovery rate (FDR) was computed to control for multiple corrections. FDR computes the expected proportion of false positive discoveries (type I errors) [130]. Here, FDR was computed using a nonparametric method described elsewhere [131]. Probes with an FDR <0.05 were considered significant.

The analysis investigating the programming effects of prenatal DEX in individuals without CAH used a different pipeline that did not employ permutation and FDR or filtering probes based on the interquartile range. Instead, for the differential methylation analysis that sought to evaluate the effect of DEX, three sets of relevant DMPs sites were identified: (a) probes with $p_{uncorrected}$ <0.01, (b) probes with $p_{uncorrected}$ <0.01 and a group difference in methylation of 5% and (c) probes with $p_{uncorrected}$ <0.01 and a group difference in methylation of 10%. Corresponding lists were computed for the treatment interaction with sex of the participant. The reason for performing the analysis in this manner was based on the following assumptions: (i) most differences in methylation between DEX-treated participants and controls would be mild; (ii) the number of investigated probes is very large and would require correction for multiple comparisons otherwise; and (iii) the aim was to determine the biological relevance of DMPs with subsequent functional enrichment analyses.

3.3.6 DNA methylation quantitative trait analysis

We further sought to investigate whether CpG methylation is associated with the severity of the disorder. Accordingly, correlations between methylation and participant phenotype and *CYP21A2* genotype were further investigated. Phenotype groups were defined and ranked by severity as control, SV CAH and SW CAH to create three groups for the correlation analysis. Genotypes were grouped based on the severity of the mildest mutated *CYP21A2* allele to create four groups for the correlation analysis. The genotype groups were defined and ranked as wt, B (n=10, p.I172N, causing SV CAH), A (n=10, G291S, p.R356Q and I2 Splice, may cause either SV or SW CAH) and null (n=7, no residual enzyme activity, including complete gene deletion, I7 Splice and p.R356W, causes SW CAH). The genetic status of the controls was not known but their mildest allele was assumed to be wt. Only patients with CAH not exposed to prenatal DEX were included in this analysis. One NC patient was excluded in that the NC phenotypic group included only this single patient. Next, confounding effects of sex and age were regressed out of the methylation data using a linear model. The residual values obtained after correction of age and sex in the linear model were applied for correlation to either phenotype or genotype using Spearman's nonparametric correlation. To estimate the significance of each CpG site for each respective analysis, a permutation-based p-value was computed in which 10 000 permutations were performed over the residuals from the linear model corrected for sex and age. Significant CpG sites whose correlation between

methylation levels and phenotype or genotype had an FDR of <0.05 were considered significant.

3.3.7 Association with cognitive and metabolic outcome

Height, weight, body mass index (BMI), glucose homeostasis, blood lipids and cognitive performance were analyzed using multiple linear regressions with CAH, age, sex and the CAH x sex interaction as predictors when appropriate (excluding age for cognition as the data were already age-corrected, see 3.2.1.). Moreover, nonparametric correlations were used to investigate the relationship between patient phenotype or genotype with metabolic or cognitive outcome. Potential confounding effects of sex and age on the data were regressed out of the data. This was achieved by using a linear model to correct metabolic outcome data for age and sex and cognitive data for sex in a linear model. The residual values obtained after correction, which are now corrected for age and sex, were applied for correlation to either phenotype or genotype using Spearman's nonparametric correlation.

Associations between methylation and previously described clinical outcomes were performed using multiple linear regression with β-values, age, sex and the β-values x sex interaction as predictors (again excluding age for the cognitive outcome data). Associations with cognitive outcome in short-term-treated healthy individuals were performed using the raw scores from the test given that the methylation in *BDNF*, *FKBP5*, *NR3C1* and *NR3C2* were associated with age and therefore needed to be corrected for this in the model. For all analyses, associations and correlations with a nominal p<0.05 were considered significant.

3.3.8 Functional enrichment

3.3.8.1 Genomic regions enrichment of annotations tool analysis

The Genomic Regions Enrichment of Annotations Tool (GREAT) was applied (GREAT, version 3.0.0, http://bejerano.stanford.edu/great) to investigate the functional relevance of DEX-associated DMPs [132]. Whereas other enrichment tools only take binding sites proximal to genes, GREAT is able to include distal sites as well [132]. Functional enrichment of DMPs was performed for DEX and DEX x sex associated DMPs from the three lists of differential methylated probes described in 2.3.5. Gene sets with an FDR <0.05 were selected. Enriched gene ontologies (GOs) from all analyses were subsequently overlapped and a GO term was considered enriched if it appeared to be significant in at least two gene set enrichment analyses. This was done to avoid threshold driven results from possibly selected false positives from the differential methylation analyses.

3.3.8.2 Enrichment analysis of disease susceptibility loci

Next, DMPs (p<0.01) were investigated for enrichments at disease-associated SNPs identified in genome-wide association studies (GWAS) (https://www.ebi.ac.uk/gwas/). This analysis was performed to investigate whether DEX may alter susceptibility to disease. The focus lies on inflammatory and autoimmune disorders in which a programming effect for

altered disease susceptibility due to DEX treatment could be plausible. These disorders were: asthma, pulmonary function, inflammatory bowel disease (IBD), ulcerative colitis and rheumatoid arthritis. A set of negative control SNPs associated with terms unlikely to be affected by DEX was also included: colorectal cancer, migraine, major depressive disorder (MDD), age-related macular degeneration (ARMD), mean platelet volume (MPV) and iron status biomarkers (ISBs). For each of these 11 sets, a negative control set consisting of common SNPs acquired from the online UCSC dbSNP (v.147) database was computed (https://genome.ucsc.edu/). These sets were selected by matching each SNP with the CpG probe density of the SNP from the GWAS sets and thereby controlling for the number of SNPs included and for CpG probe density. Enrichment was computed using the Genetic Association Tester (GAT) in four genomic bins (1 kb, 2 kb, 5 kb and 10 kb) around DMPs and SNPs [133]. Here, we focus on the results from enrichment at 2 kb in that it has been shown that most CpGs are influenced by SNPs within a 2 kb range. [134]

3.4 ANALYSIS OF BRAIN STRUCTURE AND WHITE MATTER INTEGRITY

To evaluate the effects of GC treatment in the context of CAH, the effects were explored across the brain in adult patients with CAH and then comparing the patients with population controls. Cortical thickness, cortical surface area and subcortical volumes and WM integrity were investigated using structural MRI and diffusion tensor imaging (DTI). Here, we included, from the same group as in paper I, patients with CAH and controls who agreed to undergo an MRI scan of the brain. In total, 42 patients with CAH who were not prenatally treated, 8 patients prenatally treated with DEX and 51 population controls underwent the scanning procedure. Of these, five participants (4 CAH, 1 control) did not complete the scanning procedure. Six participants were excluded because of psychopharmacological medication use (1 CAH, 5 controls). Two controls were excluded (one because of excessively large ventricles and another because of signal loss in the frontal cortex related to metallic braces). Therefore, the following analyses were based on 37 patients with CAH not prenatally treated, 8 patients with CAH who were treated prenatally with DEX and 43 controls. Three of the patients in the CAH group had NC CAH, 16 had SV CAH and 18 had SW CAH. The prenatally treated patients with CAH consisted of 1 patient with NC CAH, 1 with SV CAH and 6 with SW CAH. All participants were ≥16 years.

3.4.1 Procedure and data acquisition

The MRI scan included a structural T1 acquisition, three functional acquisitions, including a resting-state (8 min) and two task-related runs during working memory tasks (2x16 min) and a diffusion-weighted imaging acquisition. Total scanning time was about 70 min in a 90-min period, with short breaks between scans. MRI scans were acquired on a 3T MR scanner (Discovery MR750, General Electric, Milwaukee, WI, USA) equipped with an 8-channel head coil. This study investigated surface-based morphometry (neocortical thickness, surface area, volume and subcortical volumes) using FreeSurfer and voxel-based morphometry (FSL-VBM, GM volume) based on the anatomical T1-weighted image (T1-weighted BRAVO

sequence, TR=7.9 ms, TE=3.1 ms, 176 slices, voxel size: 1.0x1.0x1.0 mm) and WM integrity based on the diffusion-weighted imaging acquisition.

3.4.2 Analysis of voxel-based morphology

The anatomical T1 images for all participants were analyzed with an optimized VBM protocol [135] implemented in FSL-VBM (Douaud, Smith [136], http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM; part of the FSL tools, Smith, Jenkinson [137]). The brains were extracted using the T1 images with either the brain extraction tool (BET) with f 0.1, or with the optimized brain extraction script for the pathological brain (optiBET), depending on the quality of the extraction per participant. The images were then manually corrected for imperfections and thereafter segmented to obtain participant-specific GM maps. The T1 images were registered to standard space (Montreal Neurological Institute, MNI 152) using nonlinear registration in FNIRT (a nonlinear registration tool provided in FSL) [138, 139]. The resulting images were averaged and flipped along the x-axis to create a horizontally symmetric, study-specific GM template. To prevent the creation of a biased template, an equal number of random participants were assigned to each group in the template construction. Next, GM images were registered to the template using FNIRT and modulated to correct for local expansion or contraction due to the nonlinear component of the spatial transformation. GM images were smoothed with an isotropic Gaussian kernel (σ=3 mm). Demeaned values for age, sex and total brain volume were included as covariates. Clusters were defined using threshold-free cluster enhancement (TFCE) [140, 141]; significant clusters were identified using permutation testing with 10 000 permutations. For display purposes, bidirectional contrasts were mapped onto the MNI templates. Significant clusters were localized using the Harvard-Oxford Cortical Structural Atlas from FSL.

3.4.3 Analysis of surface-based morphometry

Cortical thickness, surface area and volume of cortical and subcortical structures were measured in a surface-based approach implemented in a default FreeSurfer pipeline (http://surfer.nmr.mgh.harvard.edu/). Processing of the T1 images provides estimates for grey-WM boundaries and segmentation for subcortical volumetric structures. Imperfections were controlled for visually and improved by two experimenters blind to the condition of the participant when necessary. The cortical surface was segmented into 148 bilateral regions of interest (ROIs) using the Destrieux cortical atlas and provided surface-based data for cortical thickness, surface area and volumes, as well as volumes of subcortical structures and total brain volume (without ventricles) based on automatic segmentation using FreeSurfer. Surfaced-based metrics were smoothed using a 10-mm FWHM smoothing kernel. Details of these procedures have been described previously (http://surfer.nmr.mgh.harvard.edu/) [142-153].

Surface-based metrics were analyzed in a whole brain analysis using the FreeSurfer Qdec application by fitting a general linear model at each surface vertex. A model with two factors, group (CAH/DEX, C) and sex (male, female) and two covariates (demeaned values for total

brain volume and age) was employed for this analysis. Multiple comparison correction was performed by Monte Carlo simulation and effects were considered significant when $p<0.05$. In addition to the whole brain analyses, a ROI analysis was performed using the 148 cortical and subcortical regions of the FreeSurfer Destrieux atlas [154]. Cortical thickness, surface area and cortical/subcortical volumes were analyzed in two- and three-way ANOVAs that included the group (CAH/DEX, C) and sex (male, female) factors. Demeaned values for age and total brain volume were included as covariates. The results were further FDR-corrected. The analyses were performed using R.

3.4.4 Analysis of tract-based spatial statistics of DTI data

Three participants (2 patients with CAH and 1 control) did not have good quality DTI images and were therefore excluded from the analysis. Thus, the DTI analyses were performed on the data from 35 patients with CAH and 42 controls. Voxel-wise statistical analysis of DTI data was carried out using tract-based spatial statistics (TBSS). Fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD) were analyzed. Eddy correction was applied to correct for eddy currents and motion using the new eddy correction method without top-up before brain extraction was performed. FA images were created by fitting a tensor model to the raw diffusion data using the FMRIB diffusion toolbox (FDT) [155]. Next, FA data were aligned into a common space using FNIRT, which uses a b-spline representation of the registration warp field [156]. A mean FA skeleton was created that represents the centers of all tracts common to the group. Each participant's aligned FA data were then projected onto the skeleton and fed into voxel-wise cross-participant statistics. The nonlinear warps and skeleton projection were also applied to the participants' MD, AD and RD images using the Tbss_non_FA script provided by TBSS tools. For each measure, a permutation based p-value was calculated (using the randomize tool in FSL and 10 000 permutations per contrast) to obtain significant group differences. Demeaned values for age, sex and total brain volume were included as covariates. A threshold-free cluster enhancement p-value of $p<0.05$ (corrected for multiple comparisons across space) was used to identify significant effects.

3.4.5 Association between structure and cognitive performance and medication dose in CAH

Observed morphological changes were further investigated by studying their association with cognitive performance and medication dosage (HC or HC equivalence in mg/m2). A linear regression model with medication dosage as predictor for brain structure and brain structure as predictor for cognitive performance with age, sex and brain volume as covariates was used.

3.4.6 Association between brain morphology and disease severity in CAH

To assess the relationship between brain structure and disease severity, cortical volume, surface area and thickness of all regions of the Destrieux atlas from FreeSurfer were

correlated with the participants' genotype (*CYP21A2*) and phenotype in the same manner as described in 3.3.6. The genotypes included here were grouped and ranked by severity as null (n=4; no enzyme activity, including complete gene deletion, p.[I172N;R356W] and p.R356W); A (n=15; p.G291S, I2 splice, p.R356Q and p.P30L with promotor conversion); B (n=15; p.P30L and p.I172N); C (n=3; p.V281L) and wt. To perform correlation analysis, volumes from ROIs were fitted to a linear model correcting for age, sex and total brain volume, from which residuals were extracted. Nonparametric Spearman's correlation was then performed to examine the relation between brain structure and genotype or phenotype.

3.4.7 Association between brain morphology and DNA methylation in CAH

Changes in gene methylation were observed between patients with CAH and controls (paper IV). More specifically, one CpG (cg18486102) located in the promotor of the *FAIM2* gene was hypermethylated, with the degree of methylation being positively correlated with disease severity. FAIM2 is a membrane-associated protein that is mainly, but not exclusively, expressed in the brain [157, 158]. Because it has been investigated as a protein protecting neurons from Fas ligand activated apoptosis [159, 160], it was relevant to investigate whether the level of methylation was associated with cortical volume, cortical thickness and surface area of all regions of the Destrieux atlas from FreeSurfer. The analyses were performed on data from 29 participants (13 CAH, 16 controls). A linear regression model was employed assuming that the degree of methylation predicts the structure of the ROIs between CAH and controls. Demeaned values for total brain volume, age and sex were included as covariates. FDR correction was applied.

4 RESULTS AND DISCUSSION

4.1 COGNITION AND PSYCHOPATHOLOGY

4.1.1 Cognition in patients with CAH

Studies on cognitive performance in patients with CAH are few and the few studies that are available have produced mixed findings. Some studies show that adults and children with CAH have lower FSIQ than controls [11, 72, 161]; however, other studies have demonstrated that intelligence, regardless of age, is not affected [73]. Still, deficits in verbal working memory have been observed, which, in turn, predict a poorer performance on spatial and arithmetic tasks [75, 76]. Impairments in cognitive abilities observed in individuals with CAH are most likely due to effects of suboptimal GC therapy or early salt-losing crises [75, 76, 161].

In paper I, we investigated cognitive performance in young adults with CAH by focusing on estimates of general intelligence, executive function, learning and memory. In general, our cohort performed within the normal range of the Swedish test norms. However, we did identify deficits in executive functioning, observed as reduced performance in the tests measuring verbal working memory (*Digit Span*, p=0.024), visual-spatial working memory (*Span Board Forward,* p=0.005; *Backward*, p=0.003) and ability to inhibit an overlearned response (p=0.002). Men with CAH also presented with reduced performance on fluid intelligence/nonverbal logical reasoning (p=0.033). When comparing patients with a null *CYP21A2* genotype with patients with a non-null genotype, the null group performed significantly worse than the non-null group on fluid intelligence/nonverbal logical reasoning (p=0.042). Our results are in line with previous studies that identified deficits in working memory/executive functions in adult patients with CAH [75, 76]. However, our findings contrast with those studies that identified effects on IQ [11, 72, 161] in patients with CAH. A plausible explanation for the discrepancy may be differences in detection and clinical management of patients with CAH between countries. Differences in IQ may therefore stem from salt-losing/hypoglycemic crises, whereas the cause of deficits in executive functioning may be primarily caused by suboptimal GC replacement therapy. This reasoning would also explain why we do not find any significant differences between SV and SW patients in that the majority of our patients were spared from neonatal salt-losing crisis owing to the national neonatal screening program and may therefore have been spared from cognitive impairments. However, we did not observe an association between current GC dose (HC or HC equivalence in mg/m2) and cognitive function. Future studies should use the accumulative dose over the life time as a more reliable predictor rather than the current dose because it may change substantially over time.

4.1.2 Effects from prenatal dexamethasone treatment

Previous reports on the Swedish cohort of prenatally DEX-treated individuals showed that those treated during the first trimester of fetal life and who do not have CAH showed reduced

verbal working memory capacity [70]. In addition, our more recent follow-up study suggests that these effects may be sex-dimorphic because treated girls showed more pronounced deficits in executive functions than boys [33]. In addition to the negative impact on executive functions, the girls further exhibited broader deficits on cognition, observed as poorer performance in tests assessing verbal and nonverbal intelligence [33]. However, when this group was assessed at adult age, no cognitive deficits or increases in psychopathology or autistic traits were identified (paper II). When cognitive performance was analyzed in a within-participant comparison with cognitive performance during childhood, we observed a possible improvement in executive function. This was observed as increased scoring on tests assessing verbal working memory (*Digit Span*, p=0.04) and ability to inhibit an overlearned response (Stroop, p=0.01). However, there was no significant improvement in verbal intelligence and the girls still scored at the same level as in childhood. In DEX-treated patients with CAH, there were too few participants to perform meaningful statistical analyses. Of note was that women with CAH, treated until term with DEX, exhibited broad deficits on cognition similar to what we observed in CAH unaffected girls in childhood [33, 162]. Because the study does not contain data from a large number of participants, additional national and international studies with longitudinal follow-ups on larger cohorts are needed to confirm (or contradict) the present findings.

4.2 BRAIN MORPHOLOGY IN PATIENTS WITH CAH

Currently, very few case versus control studies of brain structure in patients with CAH have been done. The available studies are mostly based on inspections of MRIs of single cases. As regards the available data, there are reports of an increased incidence of WM abnormalities in patients with CAH [88-91]. The abnormalities are focal and diffuse and not restricted to any particular region of the brain. There is, however, one study on brain structure in CAH using a case versus control design [87]. The study identified widespread reductions in WM structural integrity and reduced volumes of the right hippocampus, bilateral thalami, cerebellum and brainstem [87]. The study also reported a significant association between current GC replacement regimens and cognitive and CNS abnormalities, as well as reductions of choline and creatine levels in the mesial temporal lobe.

4.2.1 Structural abnormalities and total brain volume

Paper V sought to investigate whether CAH is associated with alterations in brain structure in our Swedish cohort (subgroup of the participants in study I). All scans were evaluated for structural abnormalities by an independent, blinded radiologist not involved in the study as part of general institute procedures. There were no differences in the prevalence of structural abnormalities between patients and controls.

The brain volume of individuals with CAH was about 4.23% smaller than that of population controls (p=0.048). This result remained significant after controlling for sex and age (p=0.012). However, differences in brain volume were predicted by the participant's height after adjusting for sex and age (p<0.001). Although height significantly predicted brain

volume (r=0.487, p<0.001), the observed difference in brain volume between CAH and controls cannot solely be explained by differences in height because controlling for height, age and sex using a logistic regression model still resulted in a significant group (CAH versus control) effect (p=0.019) on volume. Based on this finding, all further analyses included total brain volume as a covariate.

4.2.2 Brain structure in CAH

Using FSL's VBM pipeline to test for group differences in grey matter (GM) volume and correcting for age, sex and brain volume, we observed three significant clusters in which patients with CAH had reduced GM volume compared with controls. These clusters were in the left precuneus, right precentral gyrus and cerebellum (right Cerebellar Vermis, Crus II).

With FreeSurfer's Qdec tool for whole brain vertex-wise analyses, we observed reduced thickness of the bilateral rostral middle frontal gyrus and increased surface area of the left cuneus and right superior parietal cortex in patients with CAH. Moreover, in an analysis of regions from FreeSurfer's Destrieux atlas after FDR correction, we observed reduced thickness in the left middle frontal gyrus (p=0.004) and right superior occipital sulcus (p=0.036), see figure 3. We also observed an interaction between CAH and sex for volume of the left lateral anterior fissure (p=0.011). No differences in subcortical structures were observed between patients and controls.

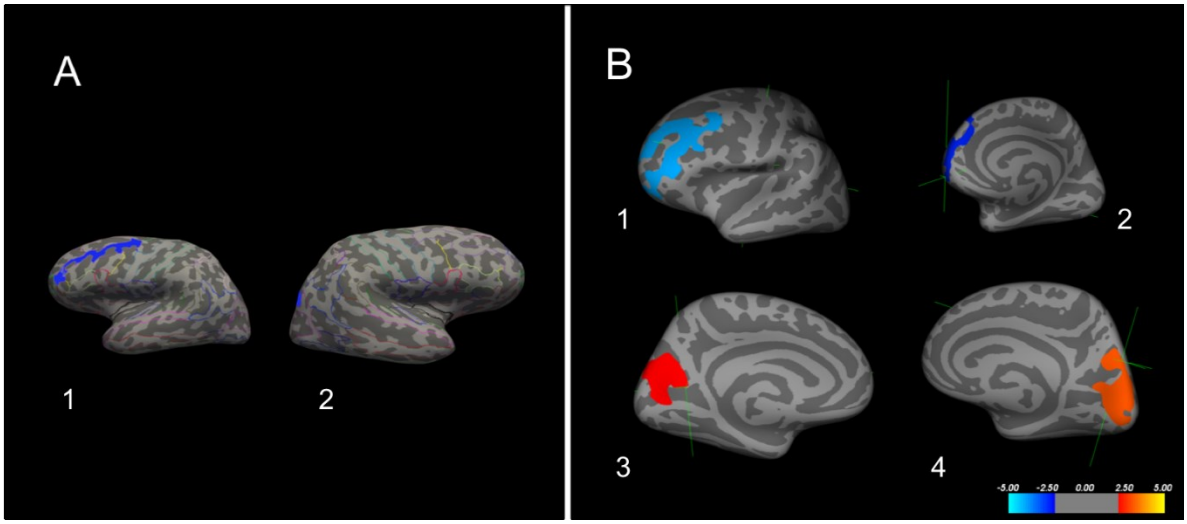

Figure 3. *Regions significantly different in structure between patients with CAH and controls. Results are displayed for the FreeSurfer Destrieux atlas analyses (A) and the FreeSurfer whole brain vertex-wise analyses (B). There are differences between CAH patients and controls for cortical thickness of the middle frontal gyrus (panel A1 and panel B1,2), and right superior occipital sulcus (panel A2). There are also group differences in surface area for the right superior parietal (panel B3) and left cuneus (panel B4). Blue colors indicate reduced size in CAH compared with controls and red increased size in CAH compared with controls; pink colors indicate statistically nonsignificant increased size (p<0.1). Figure adapted from van't Westeinde et al 2018.*

While not significant after FDR correction, we still observed alterations in structure (p>0.01). This observation might indicate further differences in brain structure but may appear as nonsignificant because of the small sample size. These alterations were reduced thickness for the left intraparietal sulcus (p=0.086) in patients with CAH, increased volume of the left inferior frontal orbital gyrus (p=0.091) and the superior temporal polar gyrus (p=0.091). There was also reduced volume of the left sub-parietal sulcus (p=0.091) in CAH.

In summary, the most notable finding was that patients with CAH showed structural alteration in the prefrontal regions involved in executive function (primarily the middle frontal gyrus and orbitofrontal cortex) and in areas of the parietal and superior occipital cortex involved in sensory integration (most predominantly the precuneus). Together, these regions comprise part of the working memory network [163]. Moreover, the precuneus is a functionally important node that supports complex cognitive processes and behavior; during rest, it also shows functional connectivity to the default mode network [164].

We also identified reductions in GM volume in the cerebellum. The cerebellum is structurally and functionally connected to the neocortex and supports activity from all major networks, including cognitive functioning [165]. The posterior Vermis and Crus II are especially associated with limbic neocortical regions and involved in affect regulation [166].

4.2.3 Effects of CAH on white matter integrity

Compared with controls, patients with CAH showed increased RD in the bilateral superior longitudinal fasciculus, see figure 4, increased MD in the bilateral inferior longitudinal fasciculus and some reduced FA in a small region of the bilateral cortico-spinal tract.

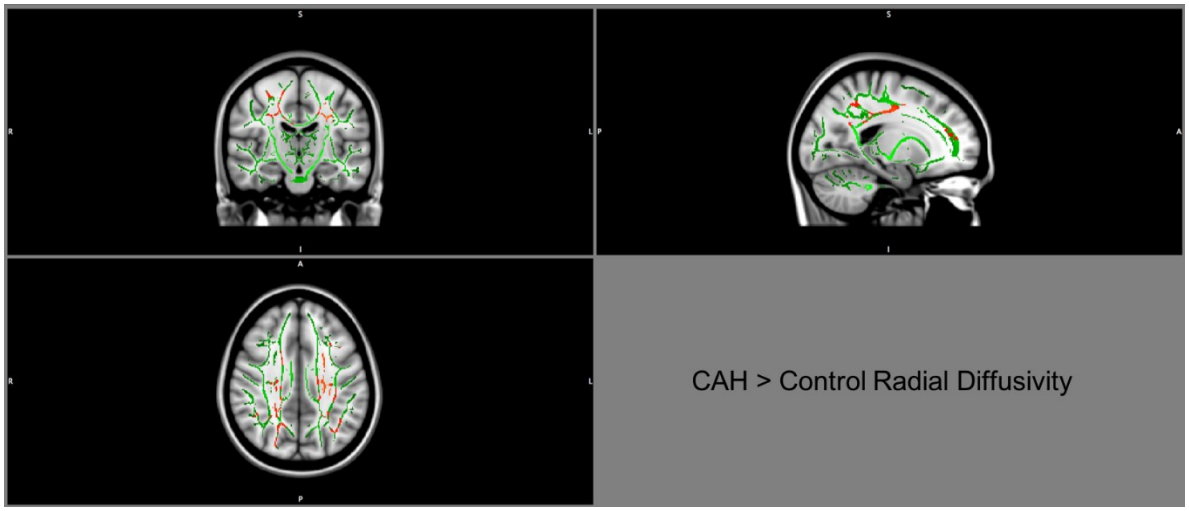

Figure 4. *Examples of results from the TBSS analyses. Patients with CAH showed increased radial diffusivity compared with controls. Figure adapted from van't Westeinde et al 2018.*

Post-hoc analysis of the group separated by sex showed that the white matter impairments were more pronounced in men. Compared with controls, men with CAH showed increased MD across most major white matter tracts, increased RD in the bilateral cortico-spinal tract and right inferior fronto-occipital fasciculus and increased AD in the right inferior fronto-

occipital fasciculus. Reduced FA in a restricted area of the left cortico-spinal tract was found in women with CAH.

4.2.4 Associations between brain morphology and medication dose, cognitive skills, genotype, phenotype and *FAIM2* methylation.

We further performed several regression analyses to determine whether GC dosage (mg/m2), cognitive skills, *CYP21A2* genotype, CAH phenotype and *FAIM2* methylation were associated with brain morphology. There was no clear association between brain structure and *CYP21A2* genotype or phenotype. However, higher doses of GCs were associated with increased volume of the left superior temporal polar gyrus (p=0.029). Particularly noteworthy was that higher doses of GCs also predicted increased FA (p=0.002) and reduced RD (p=0.023), indicating that higher dosages are associated with less damaged WM microstructure. Furthermore, increased FA predicted better visuospatial working memory performance (p=0.039) and reduced visuospatial working memory capacity was further associated with a smaller volume of the left sub-parietal sulcus (p=0.015). These findings suggest that suboptimal treatment with GCs is at least partly responsible for the cognitive deficits observed in patients with CAH. Compared with controls, patients with CAH performed worse on the visuospatial working memory tests (*Span Board Forward*, p=0.016; *Span Board Backward*, p=0.007) (see also paper I or 4.1.1).

Finally, there was a positive association between *FAIM2* promoter methylation and the surface area of the medial occipito-temporal and lingual sulcus (p=0.028) (FDR corrected). FAIM2 is an anti-apoptotic protein protecting neurons from Fas ligand activated apoptosis [159, 160]. This result indicates that DNA methylation may possibly be a mediating molecular mechanism in brain development and morphology in patients with CAH. We therefore suggest that hypermethylation of cg18486102 in the *FAIM2* promotor could result in neurons being more sensitive to apoptosis, reductions in axonal growth, or both, and are therefore associated with alterations in structure.

4.2.5 Effects from prenatal dexamethasone on brain morphology

Using the Destrieux atlas in FreeSurfer to analyze brain structure in prenatally treated patients, no differences were observed compared with controls. However, analyzing vertex-wise whole brain estimates using FreeSurfer Qdec application after Monte Carlo simulation at $p<0.05$, prenatally treated patients had reduced thickness and surface area bilaterally of a large area encompassing the parietal and superior occipital cortex (mostly the precuneus). These results are partly consistent with the observation that prenatally treated women with CAH performed worse than untreated women with CAH on most measures of cognition (paper I) [162]. Because the cognitive deficits were observed mostly in women, but the effect on brain morphology was observed in both sexes, the associations between DEX, cognitive outcome and brain structure warrant further investigation on larger cohorts. Finally, given that the sample of prenatally treated patients is very small, the results need to be interpreted with caution.

4.3 EPIGENETICS IN THE CONTEXT OF CAH

During the past decade, DNA methylation has been studied as a plausible programming mechanism for adapting long-lasting effects on health after exposure to stress or traumatic events, i.e. natural models of GC exposure. However, most researchers have investigated effects in single candidate genes and changes in gene promotors, which emphasize the need for additional investigations in this area of research using techniques with more extensive coverage, such as microarrays and bisulfite next-generation sequencing. For example, in cord blood in offspring of depressed mothers, the methylation status is increased in the GR1F promotor, and associated with an increased salivary cortisol response 3 months postnatally as a marker of altered stress reactivity in infancy [167]. Moreover, the methylation status of the *SLC6A4* (the serotonin transporter) promotor has been positively associated with exposure to stress [168], suppressed *in vivo* synthesis of serotonin [169] and linked to brain function during emotion processing [102]. There are several genes that have been associated with GC exposure and altered methylation, including *BDNF*, *FKBP5*, *TNF*, *LTA*, *SCG5* and *HSD211B2* [100, 109, 170, 171].

There are a few useful genome wide studies in this context. Using the 450K array, Weder *et al.* identified differentially methylated CpGs in saliva-derived DNA in genes implicated in stress response, neural plasticity and neural circuitry in maltreated children. The methylation status of several of these genes was associated with basal cortisol levels in children [101]. Looking back to the previously mentioned study on children exposed to PNMS during a natural disaster, Cao-Lei *et al.* were able to show that PNMS is associated with broad and functionally organized changes in the human T-cell methylome [109]. Using the 450K array, they identified specific changes in the gene programming of the immune system itself, mostly reflecting different aspects of T-cell functionality [109]. Furthermore, DNA methylation was found to be an important regulator of cytokine production in children subjected to PNMS [110] with altered levels of cytokines subsequently attributed to a shift between the levels of Th1 and Th2 cells [110, 111]. Their results are further strengthened by the finding that, in girls but not boys, higher levels of PNMS predicted a greater lifetime risk of wheezing, doctor-diagnosed asthma and lifetime use of corticosteroids [172].

Summarizing, it seems as though DNA methylation is a regulator in locking in long-term genomic programs derived from the environment of the individual, and as such, is an important factor to evaluate when investigating prenatal and life-long treatments, as in the context of CAH. Differential methylation identified in peripheral cells can be used in investigating genes relevant for brain functions (e.g., *NR3C1*, *SLC6A4*, *BDNF* and *SCG5*). The reprogrammed immune system identified in the Canadian PNMS cohort also provides insight on how stress affects peripheral cells on a genome-wide level. Children subjected to PNMS showed cognitive and behavioral alterations, but it is not known whether PNMS may cause structural changes in the brain. Alterations in peripheral cytokine levels may play a plausible mechanistic role in the formation of structural changes in the CNS, however.

4.3.1 DNA methylation in patients with CAH

We did not identify any effect on methylation from DEX or CAH. Yet, we did identify two DMPs to be correlated with a participant phenotype: cg18486102 (rho=0.58, p=0.027) and cg02404636 (rho=0.58, p=0.038). The DMP cg02404636 also correlated with genotype (rho=0.59, p=0.024). The DMP cg18486102 is located in the transcriptional start site (TSS, 200 pb upstream of the gene) region of the *FAIM2* gene and cg02404636 in the TSS1500 region of the *SFI1* gene. The CpG cg18486102 is located in a CpG island and a DNase1 hypersensitive site. They are also located in a region enriched for two histone modifications (H3K4me2 and H3K4me3) that are markers for actively transcribed promotors and transcription factor binding sites [173, 174]. The CpG cg02404636 also overlapped with a DNase1 hypersensitive site and was located near a CpG island.

The differentially methylated CpG also overlapped with regions enriched with a large number of histone modifications. Furthermore, investigating CD4+ T-cell-specific data using Ensambl (http://grch37.ensembl.org/index.html), we identified the regions for both CpGs as active promoters in this cell type.

4.3.1.1 Associations with patient outcome

In general, we did not observe any differences in cognitive measures in this subgroup of participants included in study IV. This finding may arise from the fact that children were included who still did not develop cognitive impairments. We did, however, observe higher levels of serum C-peptide in patients with CAH (p=0.044). In addition, levels of C-peptide (*rho*=0.261, p=0.044) and HbA1c (*rho*=0.274, p=0.034) were positively correlated with patient phenotype and with patient genotype (C-peptide: *rho*=0.265, p=0.044; HbA1c: *rho*=0.281, p=0.033). These results suggest that patients with a more severe *CYP21A2* mutation and clinical phenotype may be more susceptible to insulin resistance. Thus, we provide additional evidence that the severity of the disorder is associated with affected glucose homeostasis, which concurs with previous evidence [15, 175, 176]. Alternatively, we suggest the possibility that this finding is associated with GC treatment rather than disease severity. With a more severe degree of CAH, higher dosages of GCs may be necessary to achieve optimal treatment and hence this may be a mechanism of these metabolic effects.

We only identified one association between methylation and outcome, namely the CpG cg02404636, which showed a significant interaction with the sex of the participant and fasting plasma HDL cholesterol levels (p=0.035). The explanation for why we did not find additional associations may be related to the size of the cohort. Because many of our p-values were between 0.05 and 0.1, this may be a sign of a lack of power or that methylation underlies other biological factors that in turn affect outcome (e.g., brain structure) (see 4.2.4).

4.3.2 Effects from prenatal dexamethasone on DNA methylation

Using the previously described pipeline in 3.3.5, we identified three sets of DMPs associated with first trimester DEX treatment (paper III). Group differences for DMPs associated with a

main effect of prenatal DEX or an association between treatment and the participants' sex (DEX x sex) were between 0-10%. Differential methylation associated with the DEX treatment applying a $p<0.01$ probe selection criterion resulted in 9672 DMPs, corresponding to 5220 unique genes (3482 DMPs were hypermethylated and 6190 hypomethylated). Applying the second probe selection criteria ($p<0.01$ and a group difference in methylation of 5%) for probe selection resulted in 2234 DMPs sites, corresponding to 1422 unique genes (519 DMPs were hypermethylated and 1715 hypomethylated). Applying the third criteria ($p<0.01$ and a group difference in methylation of 10%) for probe selection, we identified 42 DMPs, corresponding to 24 unique genes (19 DMPs were hypermethylated and 23 hypomethylated). For probes associated with the treatment's interaction with the participants' sex for the first selection criterion ($p<0.01$), we identified 7393 DMPs with 4421 unique genes annotated (3129 DMPs were hypermethylated and 4264 hypomethylated). The second selection criteria resulted in 2786 DMPs with 1749 unique genes annotated (1613 DMPs were hypermethylated and 1173 hypomethylated). The third selection criteria gave 200 DMPs with 159 unique genes annotated (89 DMPs were hypermethylated and 111 hypomethylated). Moreover, the DEX x sex-associated DMPs had more hypomethylated probes (53.5%) than hypermethylated probes (46.5%), (Figure 2B). The percentages for DEX-associated probes were 50.3% for hypermethylated probes and 49.7% for hypomethylated probes.

The DMPs were further enriched in gene bodies and intergenic regions (all ps<0.05, Bonferroni corrected) and in open seas (regions not associated with a CpG island) (all ps<0.05). Moreover, the DEX x sex-associated DMPs were enriched in S shelves, <2 kb flanking outwards from a CpG shore (p<0.05).

These findings led us to a new question: How would these sites potentially affect gene regulation? This question was answered by performing a post hoc analysis investigating whether DMPs were enriched at specific epigenomic markers. Genomic regions for the markers were acquired from the US National Institute of Health Roadmap Epigenomics Project using data from specific CD4+ T-cells (http://egg2.wustl.edu/roadmap/web_portal/). We investigated enrichment with genomic regions enriched for the histone modifications H3K4me1 and H3K27ac (enriched at active enhancers) or H3K36me (enriched in actively transcribed gene bodies) and DNase 1 hypersensitive sites. Enrichment analyses were performed by comparing the proportion of overlapping DMPs with the distribution of probes from the 450K array using Fisher's exact test. The proportion of probes in intergenic regions and associated with the DEX x sex interaction was significantly higher than expected for H3K4me1 sites (odds ratio [OR]=2.84, p=0.0004) and for H3K27ac sites (OR=2.54, p=0.002). These findings may indicate that DEX-associated changes in methylation affect regulation of gene expression by altering the chromatin state and accessibility of regulatory elements.

We also investigated genes reported to be differentially methylated after exposure to high GC/stress levels (*BDNF*, *FKBP5*, *NR3C1*, *NR3C2*, *TNF*, *LTA*, *SCG5*, *SLC6A4* and

HSD211B2) [100, 109, 167, 169-171] to determine whether we could replicate the results from these studies. We also analyzed other genes relevant to the research questions. These are genes either involved in the regulation and maintenance of DNA methylation (*DMNT1*, *DMNT3A*, *DMNT3*, *DMNT3L*, *TET1*, *TET2*, *TET3*, *KDM1A* and *KDM1B*) or genes involved in steroid action, regulation and production (*CRH, CRHR1, SRD5A2*, *SF-1*, *HSD3B1*, *CYP21A2*, *CYP19A1*, *CYP17A1*, *CYP11A1*, *CYP11B1*, *CYP11B2, MC2R* and *POMC*).

In this analysis, we identified DMPs in genes involved in the regulation and maintenance of DNA methylation that may indicate that prenatal DEX alters the programming of the epigenetic regulatory system. Through this mechanism, DEX may have both widespread and long-lasting effects on gene regulation.

We further observed DMPs with altered DNA methylation located upstream of the transcriptional start site regions (TSS, up to 1500 bp away) and 5' untranslated regions (UTR) in several genes involved in adrenal steroidogenesis (*CRH, CYP21A2*, *CYP19A1*, *CYP11A1*, *CYP11B1* and *CYP11B2*). A possible interpretation of these results is that they reflect an adaptation in the HPA axis as a response from the prenatal DEX treatment on an epigenomic level. Fetal programming on prenatal exposure to GCs has previously been shown to affect the HPA axis. In a follow-up study, children exposed to synthetic GCs in late pregnancy and born at term showed an increase in cortisol responses to psychosocial stress, with greater effects seen in girls [68, 177]. This finding suggests an epigenetic programming of the HPA axis as a response to prenatal DEX treatment, a finding confirming with previous research [30].

The targeted genes identified in the literature to be implicated in GC exposure, stress and traumatic events all contained DMPs. Furthermore, in genes that are more relevant for brain function, we found significant associations between DNA methylation and performance in cognitive tasks. Methylation status in *BDNF* was associated with performance in the *WAIS-IV Digit Span*, *WAIS-IV Coding* and *WMS List learning, immediate recall* and *FKBP5* and *NR3C1* were associated with *WAIS-IV Matrices*. Taken together, these findings suggest that one mechanism for DEX-induced cognitive deficits may be alterations in methylation in specific neurons and in cells involved in the function of the HPA axis.

4.3.2.1 Functional enrichment

To add functional meaning to the DMPs, we performed two enrichment analyses: GREAT and GAT. The enriched GO terms were mostly related to immune functioning and inflammation but also indicated effects on other biological systems (e.g., one of the top most significant pathways is gastric acid secretion). This is a relevant finding in the sense that GCs increase gastric acid secretion and prolonged GC exposure may cause peptic ulceration or aggravate existing ulcers [178]. Moreover, an unexpected finding in our study was that the olfactory receptor activity was the top most significant GO term cluster and enriched for both the effect of DEX and the DEX x sex interaction effect. Previously, a cluster of olfactory receptor genes was identified to be differentially methylated in T-cells and in cells of the

prefrontal cortex in rhesus monkeys subjected to differential maternal rearing [179]. In addition, a recent study analyzing DNA methylation and RNA expression in patients with PTSD found differential expression of eight olfactory receptor genes or related genes in peripheral blood [180]. This result indicates that olfactory receptors may yet have unknown biological roles important for establishing the fetal programming and early life events with high GC exposure.

The effect also pointed towards an affected immune system with altered susceptibility to asthma and IBD. In the GAT analysis after FDR correction, DMPs associated with DEX showed enrichment around IBD-associated SNPs (q=0.022) and DEX x sex-associated DMPs enriched with asthma-associated SNPs (q=0.022). These results raise the possibility that when DEX alters DNA methylation *in cis*, it could contribute to the development of these disorders by altering gene expression, either on its own or synergistically occurring with disease-associated SNPs.

Taken together, a plausible conclusion could therefore be that prenatal DEX treatment creates a long-lasting program for the immune system, which could potentially lead to the development of immune-mediated inflammatory disease later in life. This contention agrees with results from the Canadian study of children who had been subjected to PNMS. Their results showed that higher levels of stress predicted a higher lifetime risk of wheezing and asthma [172]. These conditions were only observed in girls and therefore maternal endogenous cortisol exposure/GC treatment during pregnancy may affect fetal epigenetic programming in a way that may be sex dimorphic [109, 172]. This proposal also agrees with the observation that girls had broader cognitive deficits when we assessed their cognitive functions during childhood [33].

4.4 ETHICAL CONSIDERATIONS

Prenatal treatment of CAH has been performed worldwide since the mid-1980s to minimize the virilization of affected girls. However, the treatment is still considered experimental and the long-term effects are unclear. In Sweden, prenatal DEX treatment was employed since 1985 and later continued as a clinical trial (PREDEX, PI, S Lajic). Because of the recessive mode of inheritance and that only the girls are virilized, only 1 of 8 fetuses will benefit from DEX treatment and 7 of 8 fetuses (who also cannot consent) will unnecessarily be exposed to high doses of GC during early embryonic life. This is a huge ethical dilemma. Initially, the treatment was thought to be almost completely without adverse events. However, this view only lasted until the late 2000s in Sweden when Hirvikoski *et al.* observed cognitive deficits in prenatally treated participants [70]. In light of these observations, the treatment is no longer offered in Sweden since November 2010 [18].

There are also important ethical concerns regarding the design of the study on prenatal DEX. The issue is that the design is retrospective and with it comes the problem that some participants do not know that they were treated, as their parents may not have told them. This situation can be disturbing and confusing for them and disruptive for their families. This may

also be a reason for the families to refuse to participate in the longitudinal follow-up. However, not performing the follow-up and evaluation of the treatment would lead to a lack of knowledge and leave us uninformed about the long-term safety of this treatment, which is still widely used globally. Thus, it was decided that the consequences of not performing the follow-up outweighed the potential drawbacks.

All participants gave their informed consent before participation and the regional ethics committee of Stockholm approved the study. The tests and procedures were noninvasive, except for the blood samples. The participants were informed that participation was voluntary and local anesthetics in the form of plaster (EMLA) to numb the skin were used before blood sampling. After all the tests and procedures, the data carrying personal information were coded to ensure anonymity. When the data are presented, no individual can be identified from the data (anonymous data).

4.5 CONLUSIONS AND FUTURE PERSPECTIVES

In summary, our studies further elucidate the long-term consequences of prenatal DEX treatment on health in patients with CAH, as well as in individuals at risk of, but not affected by, CAH. First, with respect to the use of prenatal DEX, our studies [33, 70] show that the observed cognitive deficits, mostly verbal working memory, during childhood and adolescence seem to normalize by the time the treated individuals reach adulthood. However, the effects on verbal intelligence observed in girls do not normalize in adulthood. The young adults who were prenatally treated with DEX did not show any increase in psychopathology or autistic traits either, which is encouraging. The cohort is unfortunately relatively small and replication in larger cohorts is needed to confirm the results. Another question that can be answered by future studies is whether the normalization in verbal working memory may be related to differences in functional activation or structural changes in the brain in treated participants. We also observed that the girls treated until term who have CAH perform worse on most measures of cognitive function than prenatally untreated women with CAH at adult age. This may indicate that women with CAH are more negatively affected by DEX than CAH unaffected women and men with CAH. This may stem from treatment duration and the additional load from postnatal GC treatment.

DNA methylation levels were altered in DEX-treated participants without CAH. More specifically, it seems that in CD4+ T-cells there is evidence for an epigenetic reprogramming of the immune system, a reprogramming that may lead to altered susceptibility for inflammatory disorders (e.g., IBD and asthma) in treated participants. However, functional studies and replications in larger cohorts are necessary to confirm the results. Moreover, we did not aim to investigate if an increased incidence of inflammatory disorders exists within the current cohort of treated individuals. Together with functional studies, an increased incidence of inflammatory disorders may be investigated in future studies. Our results, however, indicated an effect on peripheral cytokines as well, (e.g., *LTA*), which accords well with studies from children exposed to PNMS [110]. Future studies should investigate whether peripheral DNA methylation in prenatally DEX-treated participants is associated with cytokine levels. Results from functional studies may provide a possible mechanism for altered disease susceptibility. We also identified alterations in methylation in genes involved in adrenal steroidogenesis, indicating altered epigenomic programming for the HPA axis. This finding may further encourage future studies to examine the effects from first trimester GC exposure on the HPA axis. If prenatal DEX alters the sensitivity of the HPA axis, this may have further consequences on immune functioning, cognition and behavior and metabolism.

For patients with CAH, we find that in adulthood executive functioning is impaired, especially working memory. These deficits seem to be related to alterations in the brain structure in hubs that are part of the working memory network, as well as damages to the white matter microstructure. We also find that around the time of the MR scan, the dose of daily GC replacement is related to white integrity, indicating that the medication is at least

partly responsible for the observed cognitive deficits. The observed structural changes in our cohort are not clearly linked to sex or disease severity. Therefore, the outcome might be the result of a complex interaction between the individual's genotype/phenotype, number of salt-losing crises and difficulties mimicking the normal circadian rhythm of cortisol across the lifespan. Moreover, *FAIM2* promotor methylation predicted the surface area of the medial occipito-temporal and lingual sulcus indicating an epigenetic factor involved in brain morphology in patients with CAH. However, further studies on larger cohorts in addition to functional studies are needed to determine whether the identified alterations affect brain structure. This complex interplay might have a pronounced effect on the sensitive circuits involved in working memory. We also suggest that the structural changes could be the result of long-term alterations in functional activation. Alternatively, changes may be due to alterations in energy supply to the brain by affected glucose levels due to sup-optimal treatment. This latter argument is supported by the evidence that the precuneus is altered in both patients with CAH and DEX-treated patients. This change may be because the precuneus is a highly metabolically demanding area, requiring around 35% more glucose than any other region in the human brain and may therefore be the first affected [164, 181]. Longitudinal study designs are needed to see how the brain is affected and how changes take place throughout development in patients with CAH and to further investigate the link between structure and function. In addition, we discovered that methylation is related to the severity of the disorder as indicated by the two identified DMPs in the *FAIM2* and *SFI1* genes. We also found that patients with a more severe genotype/phenotype may be more susceptible to insulin resistance. A plausible explanation for this finding may be that when the severity of the disorder increases and demands higher GC substation, there may be an effect on both metabolism and DNA methylation. This possibility may be investigated in the future by associating metabolic outcome and methylation with the accumulated GC exposure from treatment in patients with CAH, as this may be a more reliable variable than GC dose at the time of assessment as the dose is changed throughout the patients lifespan. This may also be relevant for the studies regarding cognition and brain structure. Furthermore, recently, treatment with modified-release hydrocortisone has been evaluated in small groups of patients with CAH [182, 183]. Results from these studies point towards that these types of GC treatments may create a more normal cortisol profile and may also be associated with a number of beneficial outcomes (e.g. metabolism) [182-184].

Finally, the studies included in this thesis have furthered our understanding on how prenatal GCs affect long-term outcome in DEX-treated individuals. We also elucidated the postnatal effects of lifelong GC replacement therapy given to all patients with CAH. We find that patients with CAH exhibit both deficits in executive functioning and alterations in brain morphology at adult age and there is some evidence that this is related to the dose of GC given to the patient. This highlights the problem of optimization of GC replacement therapy in patients with CAH and that both infra and supra physiological levels of GCs can be harmful in the long term perspective for the patient. Furthermore, the results will be of particular importance for the future use of prenatal DEX treatment given that this treatment is

still in use outside of Sweden. Although our results point towards that cognitive deficits seem to normalize by adult age, it is, however, not clear from the current study that this is a "catch-up" effect. The possibility still exist that the individuals require stronger functional activation and need to make an extra effort to reach the same performance as their untreated peers. Moreover, the finding from our study regarding DNA methylation indicates that the DEX-treated children may be more susceptible to inflammatory disorders. Therefore, our standpoint still remains that this treatment should not be a part of the therapeutic arsenal for CAH. However, for meaningful meta-analyses to be performed, more studies are needed to confirm our findings. With additional studies and evidence, stronger conclusions can be drawn to support and inform the clinicians on how to better manage patients with CAH.

5 ACKNOWLEDGEMENTS

First of all, I want to extend my gratitude to my supervisors: **Svetlana Lajic**, **David Gomez-Cabrero**, **Anna Nordenström** and **Michela Barbaro**. Without your unwavering support and encouragement, my PhD at Karolinska Institutet would not have been possible. Each and every one of you have introduced me to new and different areas of medical science and helped me to become more enthusiastic and committed to my own research. **Svetlana** and **Anna**, you have been crucial in introducing me to pediatric endocrinology. **Svetlana**, you also helped me a great deal in the lab initially, although you already had a lot of things to do on your end. David, through your teachings, I became highly motivated to learn more about bioinformatics, epigenetics and programming. Last but not the least, **Michela Barbaro**, if you would not have taken me in to perform my bachelor project in your group, then I would most likely not be active within science today. The time spent with you while writing my bachelor and master projects made me both curious and fascinated with medical science and research. I extend grateful thanks to all my supervisors for their professionalism, patience, guidance, wisdom and faith in me.

I would also like to express my gratitude to my co-author and colleague, **Tatja Hirvikoski**, for taking the time to teach and help me with the neuroscientific aspects of my projects.

I also want to give my heartfelt appreciation to the Medical Students' Association at Karolinska Institutet (Swedish: **Medicinska Föreningen)** and all the people I met and worked with there. You created a warm and welcoming environment that, for a lonely student coming from the forest southwest of Stockholm, has been invaluable.

Finally, I want to express my profound gratitude to my **family**, **friends** and **colleagues**, both in and outside of academia. You have all contributed tremendously in various ways and my PhD would have been a very different (and much more difficult) experience without you. You have all been extremely supportive during my PhD studies and I am so grateful to have you in my life.

Thank you all for accompanying me during this thesis work.

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